Seasonal timing in a changing climate

Schaper, Sonja Verena

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Seasonal timing in a changing climate

The physiological basis of phenotypic plasticity and its evolutionary potential

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Beoordelingscommissie:	Prof. dr. M. Hau
	Prof. dr. J. Ellers
	Prof. dr. J.M. Tinbergen

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“The only thing constant in life is change”

François de la Rochefoucauld (1613-1680)

Chapter 1

General Introduction

Evolution in action

Over evolutionary time, all organisms on this planet have been constantly adapting to an ever-changing environment and this process is still ongoing. Habitats of plants and animals change continuously in response to climatic changes, affecting the community of all interdependent living organisms inhabiting them. Even over relatively short time-scales, changes in the abiotic environment can lead to a loss of competitive advantage and finally the extinction of those species that are not able to adapt to the new conditions. Ambient temperature is particularly important for structuring biological systems and thus over evolutionary time the environment has posed a constant shifting 'thermal challenge' to all organisms by affecting energy utilization, growth and also reproduction and survival (Clarke 1996). At the individual level, coping with climate in general is thus a question of tracking continuous change, more than adapting to a mean temperature (Clarke 1996).

Environmental changes can happen on different scales, ranging from subtle and long-time to sudden and erratic. They can be predictable to a certain degree and these predictable changes range from seasonal to lunar to daily periodicities. To visualize these time-scales, just think of a macroalga growing in the intertidal zone of the Wadden sea. Due to the changing tides, the plant repeatedly endures extreme daily changes in light, moisture, temperature and salinity. It also experiences altering conditions induced by the lunar cycle, which influence the duration of flooding and the height of the water body submerging the plant, but is also exposed to the seasonal changes in light conditions and water temperature. The alga experiences these changes repeatedly during all stages of its own lifetime, but its descendants and the whole population will be exposed to changes in mean water temperature and sea level that are caused by climate change in the long run.

When we investigate a species' contemporary resilience to climate change, it gives us the chance to examine general principles in action that have determined the success of organisms over long time-scales. These include morphological, physiological and behavioural reactions to temperature changes on a time-scale significantly shorter than the life-span of an individual, including phenotypic adjustments and migrations. However, the rules underlying these adjustments might also fundamentally differ from the processes underlying long-term phenomena such as shifts in the distribution of populations, adjustments through evolutionary temperature compensation, or even extinctions in response to subtle rates of climate change (Clarke 1996). In this dissertation I concentrate on the capabilities of a small song bird to respond to an environment that changes over the lifetime of an individual. I take into account the possibility of micro-evolutionary adaptation in response to long-term climatic changes.

The concept of phenotypic plasticity

The range of environmental conditions in which an organism can grow and eventually reproduce, and thus the width of its niche, can define its success in a changing environment. The success lies in the ability to physiologically regulate its state in response to environmental variation to sustain homeostasis and especially in animals behavioural adaptations widen the range of environmental conditions in which an animal can grow and reproduce. Many organisms are able to change their phenotype in different environments and as most environments are changeable over short time scales, phenotypic plasticity is a key process within evolutionary biology. It is defined as the property of a given genotype to produce different phenotypes in response to distinct environmental conditions (Pigliucci 2001). The relationship between the phenotypic trait and the environmental variable affecting its value is termed a reaction norm: the range of phenotypes produced when an individual is exposed to different environments, such as a temperature gradient. Behavioural, physiological and morphological plasticity are all essentially similar genotype-environment interactions on different levels (Pigliucci 2001).

Factors that favour or constrain the evolution of phenotypic plasticity are yet not well understood (DeWitt et al. 1998; Pigliucci 2005; van Kleunen & Fischer 2005; van Buskirk & Steiner 2009; Auld et al. 2010). In principle, organisms that are capable to fine-tune their phenotype to the immediate environment, or to a future environment where selection takes place, should benefit from a higher overall performance. However, there are also costs and limits to the ability to be plastic which need to be acknowledged. If these constraints are too severe, individuals may benefit more from mechanisms that reduce environmental influences on trait expression (environmental canalization).

Limits to phenotypic plasticity involve four mechanisms: 1) lag-time responses to environmental change, 2) restrictions of the rate of change if the response to one environmental factor limits the response to a second factor, 3) epiphenotypic limits, where environmental changes detected late in ontogeny do not allow for a sufficient reorganization and 4) a history of plasticity that (physiologically or energetically) does not allow for further changes (DeWitt et al. 1998; Auld et al. 2010).

Costs, on the other hand, arise mainly as maintenance costs of sensory and regulatory systems, but also include elevated developmental costs for the expression of a given (morphological or physiological) trait, compared to a non-plastic individual. Ecological costs, such as sampling the environment, which requires time and energy and is risky in the presence of a predator, should also not be forgotten. Additionally, if developmental processes of plastic individuals are more sensitive to the environment during ontogeny, these developmental-instability costs might potentially lead to imperfect matching between a more extreme phenotype and a future environment.

Finally, intrinsic genetic costs emerge from linkage, pleiotropy and epistasis of genes involved in the ability to be plastic and pose costs that might reduce the fitness benefits of plastic individuals in comparison to non-plastic ones (DeWitt et al. 1998; Auld et al. 2010).

Usually, the genotypes resulting in the most plastic phenotypes also show the most extreme trait values (Auld et al. 2010) and thus the costs of plasticity are in general hard to distinguish from costs also paid by non-plastic individuals showing extreme phenotypes. In addition, the co-linearity between trait value and plasticity can be environment-specific, which means that comparing the performance of plastic and non-plastic individuals in a narrow subset of environments is questionable (Auld et al. 2010). Possibly due to these limitations, only weak costs of plasticity were identified in the majority of studies (van Buskirk & Steiner 2009). This should not be surprising, as natural selection will have worked against cases where these costs outweigh the benefits of plasticity.

Phenotypic plasticity will thus only evolve under four conditions: 1) populations must experience heterogeneous environments that affect the fitness of alternative phenotypes; 2) there must be reliable environmental cues; 3) no single phenotype can experience superior fitness in all environments; and 4) costs of plasticity must be moderate (Via & Lande 1985; Relyea 2002).

Phenotypic plasticity allows persistence of a population under novel environmental conditions within a certain range, thereby leaving more time for micro-evolution to take place and thus also plays a significant role in adaptation of the mean trait value (Pigliucci 2005). In addition, even though we commonly observe a linear slope of the mean reaction norm of a population, individuals within the population may vary in the steepness (Nussey et al. 2005) or shape (Reed et al. 2009) of their reaction norms and if there is genetic variation in and selection for the steepness of the reaction norm this might enhance the rate of adaptation in response to a shifting environment (Visser 2008).

If an environment is continuously changing directionally, individuals that show a high degree of plasticity to this environmental component will initially have a selective advantage, but most likely in the long run only a micro-evolutionary change in the mean trait value can lead to a better adaptation. Organisms can thus adapt to an environment that, on top of small-scale variation, is shifting directionally for a long time by two mechanisms: micro-evolutionary changes in the degree of phenotypic plasticity, which is especially adaptive if a high level of variability is superimposed on the general linear trend, or micro-evolutionary changes in the mean trait value itself, which is selected for if the directional trend is more influential than stochastic changes.

Plasticity in the organization of life histories

For short-lived organisms, encountered environmental changes might affect the majority of their existence and phenotypic plasticity will mostly involve non-reversible developmental changes as a reaction to the current prevailing environment. Such is the classic case of *Daphnia* water fleas that adapt their morphology during development and grow spines in the presence of predator cues to reduce predation risk (Woltereck 1909; Krueger & Dodson 1981). In contrast, more long-lived species will possibly need to react repeatedly to environmental changes throughout different stages of their life. They will make decisions on when to reproduce and how many offspring to raise in each reproductive event and the optimal decision rules will vary largely with the encountered environment at the time of decision-making. Sometimes, this environment will differ from the environment of selection, in which case predictive cues are taken into account in the decision-making process to predict conditions later on (Visser et al. 2004). Life-history theory investigates the outcome of these individual decisions under the assumption that optimal strategies are those that result in the largest possible number of surviving offspring in the environment of selection over the lifetime of an individual. In many cases, key events in the life of an individual, such as reproduction or migration in birds, are repeatedly undergone every year and have to be plastically fine-tuned to the environment. Because the seasonal timing of many of the life-history stages have important fitness consequences, the precision of the timing mechanisms will be under strong selection. Endothermy combined with a high body temperature has enabled birds and mammals to be largely physiologically buffered against seasonal temperature fluctuations, but the seasons still drive the organization of their life histories.

Seasonal timing and predictive cues in birds

Most birds of temperate zones are strictly seasonal breeders and show distinct cycles of breeding, plumage moult and in some species migratory periods. Life-history events that require a lot of energy are timed to periods when the abundance of food items allows for the gathering of sufficient resources that exceed the daily requirements. In these periods, for example, the energy-intense production of eggs, raising of young, or growth of new feathers can be sustained. As these consecutive events have to be synchronized with the seasonal phenology, seasonal changes have to be anticipated. In the case of avian breeding, preparations for reproduction have to be initiated months in advance, so that the growth of reproductive structures, mating and nest building is finished before environmental conditions actually allow for the energy-intense production of eggs. But how is this precise seasonal timing plastically tuned to the local environment?

Photoperiod constitutes the primary predictive cue that regulates the lives of most organisms on earth, both on a daily and a seasonal basis (Bradshaw & Holzapfel

2008). At a particular latitude, changes in photoperiod are highly predictive over the year. The steady increase of light hours per day is thus a reliable predictor of date and is used as a strong stimulus to anticipate seasonal climatic changes and induce seasonal behaviour, also in birds. Additionally, like most organisms, the majority of birds depend on a circannual clock, or an internal 'seasonal calendar' determining motivational state (Helm 2009) that is entrained by photoperiod (Gwinner 1986; Gwinner 1996). When days get longer, hormonal changes induce the growth of the avian gonads from their regressed winter state. When the gonads are functional, they excrete steroid hormones that in turn feedback on the brain and induce behaviours such as courtship and nest building and thus facilitate the start of laying. The timing of each of these physiological changes, which are linked to reproductive decisions, has to be carefully synchronized with the appropriate time of year. However, as climatic conditions are quite changeable between years, seasonally breeding birds make use of so-called supplementary cues (Wingfield & Kenagy 1991; Dawson 2008) that allow for a more accurate prediction of the seasonal progress and a fine-tuning of the onset of breeding.

In warmer springs, seasonal events, such as the sprouting of trees or the appearance of certain insects are advanced and early breeding is also advantageous for birds. Temperature is as a major driving force of biological seasonality in temperate zones (Parmesan 2007) and is believed to be the most influential environmental cue to fine-tune seasonal breeding behaviour of birds to local conditions. Yet, defining the components of an experienced temperature profile that induce laying is still a challenge (see **Box 1.1**). Besides climatic components, the development of the vegetation itself could be perceived as a more immediate predictive cue. We traditionally score the onset of spring from a number of phenological observations, such as the first blossoming of apple trees, or appearance of butterflies, which happens in direct response to recent temperature conditions. It is thus possible that birds make use of these secondary temperature-related cues as well, but as they are highly correlated with temperature, it is not possible to separate the influences of phenological and temperature cues under natural conditions. Even food availability itself can be seen as a predictive cue. Supplementary cues are taken into account by birds because they have a predictive value for future events. As an example, the increase in temperature might indicate the upcoming availability of insect prey for a bird in the temperate zone woodlands, whereas a bird in the Australian desert might consider the beginning of seasonal rains, which induce a flush of flowering grass as an adequate stimulus (Zann et al. 1995). Cues are thus highly context-specific and vary even between closely related species, dependent on the environment they inhabit.

Additionally to these environmental cues, individuals in a population can possibly adjust their own behaviour in response to social information from conspecifics in their surroundings. For example, the first male birds that start singing in a population

could induce an advancement of courtship behaviour in other males (Helm et al. 2006). Lastly, it is possible that experience from previous years, or learning, might influence the decision of an individual in when to reproduce in coming years (Grieco et al. 2002).

In this dissertation, I focus on the way individual birds react to environmental cues for their reproductive timing and among these on cues provided by ambient temperature directly. However, I also investigate the inheritance of cue sensitivity with respect to photoperiod and temperature. This is of special interest in view of current climate change, which might shift selection pressures on the way animals should time seasonal reproduction in response to environmental cues.

Box 1.1: The thermal environment

Organisms usually encounter a range of environmental temperatures during their life to which they have to respond adaptively. Yet, investigating the effects of ambient temperature on the physiology and behaviour of an organism is not straightforward. This is partly due to the correlation of temperature with many other components of the environment, including vegetation phenology. Environmental temperature varies in time and space, changing both continuously and unpredictably and even rates of changes are variable. The solar cycle, heat exchange between ocean, land and atmosphere and other physical processes generate regional variations in local climate on many time scales (van den Hurk et al. 2006). Inter-annual variation is largely driven by atmospheric processes, such as the North Atlantic Oscillation in the Northern hemisphere or the El Niño Southern Oscillation in the Southern Hemisphere (Clarke 1996). Besides these long-term patterns, there are also seasonal and diurnal changes that determine a specific temperature environment. While a short-term temperature trend can be to a certain extent predictive of long-term developments, for example seasonality, the pattern is overlaid by erratically fluctuating small-scale temperature variation over successive days or weeks.

Defining the period of interest in relation to a particular event, such as the onset of breeding in birds, requires careful thinking about the predictive or physiological relevance of a particular temperature cue. While, for example, the intensity of incubation behaviour is likely adjusted in response to present temperature conditions, the onset of breeding is more likely the outcome of a long-term predictive temperature integration. Two common approaches, the temperature sum approach and the sliding window approach, both aim at collapsing temperature patterns of a period of interest into one value, such as the mean temperature of a time-span pre-laying, and correlate this value with the event of interest. However, it is mostly unclear over which

period predictive temperature needs to be averaged, as responses to a given temperature or temperature change can vary markedly between species depending on their ecology (Clarke 1996) and even between different life-history stages of the same species. Since our knowledge of the mechanistic action of temperature cues is still very limited, correlating a phenological event with the mean temperature over a fixed period has clear disadvantages, as relevant information might be lost from a temperature pattern.

Furthermore, temperature differs largely over the course of the day and thus it can be expressed as the daily temperature range, minimum, maximum, or just mean temperature over a day, or a variable amount of time. Another problem is posed by the fact that the experienced temperature could be perceived as absolute temperature, or temperature relative to own body temperature (which, however, will be fairly constant in homeotherms). Under natural conditions, other climatic variables, such as wind speed or humidity could further influence the apparent temperature an individual perceives. Furthermore, when investigating temperature effects on physiology, it is impossible to distinguish direct physiological thermal effects, such as the acute temperature-dependence of biological rate processes, or temperature effects on energy expenditure due to thermoregulation, from effects that come about by behavioural changes in response to temperature cues. It is thus obvious that setting up controlled experiments that investigate effects of temperature on behavioural decisions of animals is quite a challenge.

Climate change and predictions of the future

Organisms are in general capable of dealing adaptively with long-term environmental changes, however, the current rate that is driven by anthropogenic climate change is by far exceeding historical accounts (IPCC Core Writing Team 2007a). On top of the normal interannual fluctuations in seasonal climate, we currently experience an ongoing linear long-term warming trend. This global trend of on average 0.13°C temperature increase per decade for the last 50 years (up to 2005), is nearly twice that for the past 100 years (IPCC Core Writing Team 2007a). In Europe the average temperature is predicted to increase slightly faster than this world average. In the Netherlands the average temperature has risen by 1.6°C since 1900. According to the latest calculations of the Royal Netherlands Meteorological Institute, the months February and March have seen the largest increases in temperature (van den Hurk et al. 2006). On top of a general warming trend, cold days, cold nights and frosts have become less frequent, while the number of hot days and nights have significantly increased (IPCC Core Writing Team 2007a). In the northern hemisphere there is also a decrease in diurnal temperature variability during the cold season associated with the decrease of sea ice and snow with increasing temperature (IPCC Core Writing

Team 2007a). Due to the delayed response of the climate system, these changes will persist for a very long period of time, even considering a reduction in the emissions of greenhouse gases in the near future.

Unlike the slow changes in the set of environmental variables that species experience, for example during range expansions, these relatively rapid changes have the potential to disrupt the connection between predictive cues and phenology. The fourth report of the Intergovernmental Panel for Climate Change (IPCC) clearly stresses that many natural systems are being affected by regional climate changes and particularly by temperature increases: "*There is very high confidence…that recent warming is strongly affecting terrestrial biological systems, including such changes as earlier timing of spring events, such as leaf-unfolding, bird migration and egg-laying; and poleward and upward shifts in ranges in plant and animal species*." Clear evidence of these phenological shifts is abundant (reviewed by Parmesan & Yohe 2003; Root et al. 2003; Cleland et al. 2007; Parmesan 2007; Thackeray et al. 2010). Many of these observational studies focus on changes in mean avian laying date over time (Crick et al. 1997b; Crick & Sparks 1999; Dunn 2004), but these changes should be taken as an indication of possible shifts in other systems that are less visible to the human eye. However, the shifting rate in phenology is not similar between species of the same taxonomic group and even differs within a single geographic location (Dunn & Winkler 1999; Sanz 2002; Sanz et al. 2003; Visser et al. 2003; Both et al. 2004). For European populations of great tits, Visser et al. (2003) found that despite a general advancement in breeding phenology (between 1979-1998: 3.1 days per decade), the rate of change was quite variable among populations. Most of these observed shifts in avian breeding or migration times are linked to changing mean temperatures (Crick & Sparks 1999; Both et al. 2004; Dunn 2004) or large-scale atmospheric phenomena, such as the North Atlantic Oscillation (Sanz 2002; Lehikoinen et al. 2004; Gienapp et al. 2008). Even though the general trend shows that timing is advancing, the question is if these shifts are sufficient (Visser & Both 2005; Visser 2008)?

As species within a community are unlikely all shifting with the same speed in response to temperature changes (Visser et al. 1998), the timing of dependent phenological events, such as the development of plants, emergence of insects and reproduction in insectivorous birds, can drift apart. Indeed, in the majority of climate change-related shifts, these have led to a phenological mismatch (Visser et al. 1998; Harrington et al. 1999; Stenseth & Mysterud 2002; van Asch & Visser 2007), which often leads to substantial shifts in food web dynamics and potentially affects species abundances and community composition, biomass, or even ecosystem functioning (Parmesan & Yohe 2003).

In practice, secondary consumers advance their seasonal timing less than primary producers and primary consumers in a range of terrestrial, marine and freshwater ecosystems (Thackeray et al. 2010), partly due to the fact that plants and invertebrates

experience strong direct temperature effects on their development times, whereas homeothermic vertebrates are less responsive to temperature on a physiological level. For example, we know that terrestrial invertebrates in the UK shifted their phenology stronger (4.1 day per decade) than terrestrial vertebrates (2.6 days per decade, Thackeray et al. 2010). In 7 of 16 cases for which data on the shift in phenology was known for a bird species (mostly small woodland birds, such as the great tit) and its insectivorous prey, climate change induced a phenological mismatch (Visser et al. 2011b). It is thus quite likely that changes in local climate patterns will affect population viabilities in birds by inducing sub-optimal shifts in phenology, as mismatched reproduction has substantial consequences for reproductive success and/or survival (van Noordwijk et al. 1995; Thomas et al. 2001; Nussey et al. 2005; Charmantier et al. 2008), also mirrored in population trends (Both et al. 2006; Møller et al. 2008; Both et al. 2010). These strong responses to small changes in climate patterns visualize clearly that we need to assess how species and communities are able to cope with these, from an evolutionary point of view, rapid changes. Even if species are shifting their seasonal timing in response to a changing climate, the response might not be sufficient (Visser & Both 2005), and additionally, shifting one component in the life-cycle of an individual might have consequences for the timing of another, possibly temperature-independent, stage that also needs to be phenologically matched to the environment (Crozier et al. 2008; Miller-Rushing et al. 2010). A typical example would be the accommodation of the avian spring breeding period and autumn moult, which might shift relative to each other within the crucial period of sufficient food availability, as these two life-history stages will be regulated by different predictive cues.

Besides phenological shifts, species might also circumvent climatic changes in one location by changing their geographical distribution and thus retain their 'climatic envelope' (Thomas et al. 2004) at another location which now resembles the previous one. When we apply this concept to seasonal timing in birds, however, the necessary conditions of a comparable photoperiodic and temperature regime are quite specific. A range shift would thus again require micro-evolutionary changes in response to the local environment, especially an adaptation to the 'new' set of cues that predict the encountered food web. This might take longer than necessary under current climate change. Again, the question is not whether a species responds to climate change or not, but whether these changes restore the synchrony with its environment, e.g. its prey phenology, and thus mitigate a decrease in fitness.

If we ourselves want to predict the future, it is crucial to identify species responses to climatic scenarios and investigate proximate mechanisms underlying these processes. We need to identify how climatic and possibly biotic cues are affecting timing decisions made by individual animals over their whole life-cycle and how these decisions ultimately affect fitness. Additionally, we need to investigate the potential

for a micro-evolutionary response to the long-term directional change, as shifts in phenology due to phenotypic plasticity alone will most likely not be sufficient. Only this knowledge will allow us to look ahead and estimate population-level responses to climate change (Visser 2008).

Box 1.2: Study system

Great tits (*Parus major* L.) are small passerine birds that inhabit woodlands throughout large parts of Eurasia and parts of North America (Cramp & Perrins 1993). Their abundance and wide distribution indicates that the species is able to breed in a wide range of environmental conditions. Females start egg-laying around April/May in Central Europe. Despite large differences in the onset of breeding between populations from different latitudes, or in lowland versus montane habitats, great tits are strictly seasonal breeders. The female lays one or more clutches of about 6-12 eggs into treeholes or other cavities and thus great tits willingly accept nest boxes to breed. One egg is laid per day, usually in the early morning. Incubation of the completed clutch takes another 12 to 15 days and is exclusively done by the female, whereas both parents feed the young during the nestling phase, which lasts around 18-21 days (Gosler 1993). After fledging, the parents take care of the young for another 1-2 weeks and after independence of their first brood the parents might start a second clutch (Cramp & Perrins 1993).

Great tits are predominantly insectivorous, but they consume a wider range of food items in the winter months and are especially dependent on beech (*Fagus sylvatica*) nuts to survive the winter. For their chicks, lepidoptera larvae are the most digestible source of nutrients and form 60-95% of their diet (Gosler 1993). In central Europe, the principal food for great tit nestlings consists of several species of moth larvae, predominantly the winter moth *Operophtera brumata*, which feed on the freshly developing leaves of a range of tree species, but preferentially pedunculate oak (*Quercus robur*) in spring. The life-cycle of the winter moth is highly seasonal itself: Imagines eclose from their pupae between October and December, after which they mate and lay their eggs on the branches of the host tree. These eggs then develop slowly over winter until they hatch in April, synchronized with the bud burst. As the females are wingless and in principle lay their eggs on the same tree they were born from, matrilines are locally adapted to the phenology of the specific host tree. However, the phenology of egg hatching is also strongly affected by temperature, as shown both in the wild and under controlled conditions (Visser & Holleman 2001; van Asch et al. 2007) and mean egg hatching date has advanced in the past 25 years due to climate change even more than the bud-burst of oak trees (Visser & Holleman 2001). Because of the short

availability of tender oak leaves, a sufficiently high abundance in caterpillars for the great tits is restricted to a narrow time window of about two weeks in spring. Due to the biomass peak in this period, rearing conditions for great tit chicks are optimal and most of the clutches are raised.

The great tit population used in this study is situated at the Hoge Veluwe National Park (the Netherlands) and has been continuously monitored since 1955. The standardized monitoring creates a wealth of knowledge on breeding parameters and population dynamics. The close observation of all breeding pairs in the population, the continuous ringing of parents and nestlings with unique ring numbers and partly the genetic verification of paternity by the social parents from blood analyses allows us to create a pedigree for all individual birds in the population that have been born in the Hoge Veluwe. The large dataset not only allows investigating the effects of recent climate change on timing of breeding and population dynamics (Figs. 1.1.,1.2), but also facilitates the selection of birds with specific criteria for further experiments. The study population is particularly interesting as birds apparently do not advance their egg laying enough in response to climate change to stay synchronized with the local food peak (Visser et al. 1998; Visser et al. 2006), in contrast to another long-term-monitored population in Wytham Woods near Oxford, United Kingdom (McCleery & Perrins 1998). Since the first interest in and observations of how breeding biology of these birds is regulated by spring temperature (Kluyver 1952; Perrins 1965; van Balen 1973; Schmidt 1984), great tits have become a wild model species for research on reproductive timing. However, most insight so far comes from natural observations and studies on the proximate mechanisms underlying the between-individual variation in egg laying dates are currently lacking, which inspired the present thesis project.

This thesis

Research goal

The principal aim of this thesis was to investigate the causes of individual variation in the timing of reproduction in response to environmental cues in great tits (**Box 1.2**). Previous work has focused on differences in mean laying dates of particular bird populations, for example living in different habitats (Lambrechts et al. 1997b), at different latitudes (Slagsvold 1976; Silverin et al. 1993) or elevations (Perfito et al. 2004), but mostly focussing on between-year variation. However, underlying these population means are decisions of individual birds and an intriguing question is why individuals of the same population experiencing a fundamentally similar environment differ greatly in the time they initiate breeding in a given year. Several

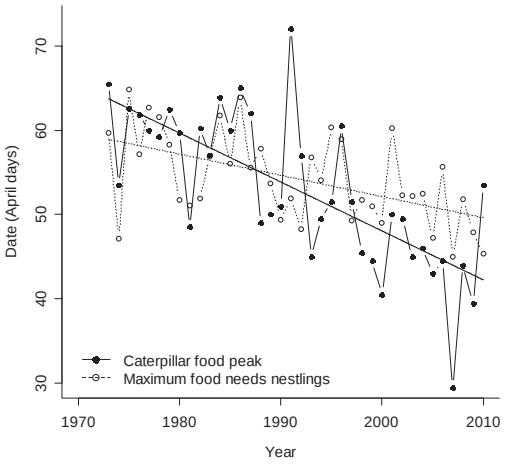

Figure 1.1: Changing spring phenology between 1973 and 2010. The date of peak caterpillar biomass, determined from frass-fall samples collected at the Hoge Veluwe, has advanced significantly by 22 days or 0.58 days a year (closed circles, solid line). Even though great tits have also advanced their onset of reproduction significantly by 9.5 days or 0.25 days a year, the period of maximum food needs for the nestlings, here approximated as one month after the mean laying date (open circles, dotted line), is currently mismatched with the period of maximum food abundance.

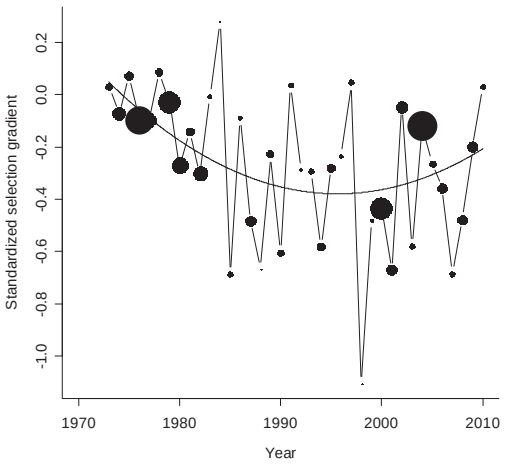

Figure 1.2: Natural selection on the timing of reproduction for years 1973 to 2010. The more negative the selection gradient, the stronger the selection for early laying in a particular year, i.e. the more offspring will an early-laying female leave in the population compared to a late-laying female. Standardized selection gradients (Lande & Arnold 1983) were calculated from the slope of a regression of the relative number of recruits (divided by the mean in a given year) against standardized laying date (laying date minus mean laying date of that year, divided by the standard deviation). This fitness measure does not take the survival prospects of the female into account. The size of the circle indicates the weighing of a particular year for the calculation of the regression line, based on the standard error around the selection gradient slope.

breeding parameters, such as the onset of laying, vary within a population by a similar amount as between years (Nager 1990). Furthermore, individuals show remarkable consistency in their timing relative to the population mean, which is due to the fact that in recent warm springs individuals with a steeper reaction norm in response to environmental cues are always earlier. We can thus identify individuals characterized by an early-laying pattern and individuals characterized by a late-laying pattern. This variation is present even though reproductive success declines over the breeding season (Perrins 1970; Verhulst & Tinbergen 1991; Verhulst et al. 1995) due to higher recruitment rates of early fledglings (Verboven & Visser 1998; Visser & Verboven

1999). Moreover, we know from previous field studies that the degree of phenotypic plasticity underlying laying dates is heritable (Nussey et al. 2005, but see Husby et al. 2010), offering exciting possibilities by comparing relatives. Understanding the physiological basis of individual variation in the onset of reproduction is crucial if we want to identify how the population as a whole responds to ongoing climate change, either via (micro-evolution of) phenotypic plasticity or micro-evolution of the mean trait value, i.e. the elevation of the reaction norm.

Differences in the timing of egg-laying between females of a population can be explained by two, not mutually exclusive, hypotheses. Both aim to explain why many birds do not advance their reproductive timing with a sufficient amount to remain synchronized with the timing of the caterpillar biomass peak.

1. The *cues hypothesis*: Females differ in the way they perceive and transduce predictive environmental cues into a behavioural decision and thus perceive different optimal laying dates. According to this hypothesis, the consistency in their laying early or late in relation to the population mean comes about because individual females are more or less sensitive or reactive to predictive cues.

2. The *constraints hypothesis*: Females aim to lay at the same optimal (early) date, but because of energetic costs it is only for females in good condition adaptive to lay at that time, whereas for individuals in worse condition these costs are too high to be compensated by the benefits of a better synchronisation. According to this hypothesis, the consistency in their laying in relation to the population mean thus comes about because individuals differ in condition, such as the ability to gather resources, claim a better territory or convey energy from self-maintenance to reproduction.

This thesis is focused on the female's use of predictive environmental cues. As temperature is the driver behind most between-year differences in seasonal development in the bird's environment, the main aim was to investigate direct effects of temperature on the timing of avian reproduction. As the timing of consecutive life-cycle stages is dependent on each other and has to be adapted to seasonal changes as a whole, I investigated effects of temperature cues on the complete breeding cycle, including pre-laying reproductive physiology, but also timing of gonadal regression and onset of moult. Furthermore, I investigated the possibility for micro-evolutionary responses to a changing environment by comparing genetically related individuals in their response to environmental cues.

Outline of the thesis

In **chapter 2** we introduce the concept of seasonal timing in more detail and discuss its application in different contexts. We exemplify how biologists from different disciplines, ranging from evolutionary ecologists, physiologists to chronobiologists and molecular geneticists, approach the phenomenon of seasonality, its proximate regulation and ultimate consequences for fitness. We stress the importance of integrating mechanisms underlying seasonal timing in a framework considering fitness consequences of the plastic phenotype. This is especially crucial if the environment shaping the phenotype will differ from the environment of selection.

Chapter 3 and **4** are centred around the main question of this dissertation, how reproductive timing is fine-tuned in response to temperature cues. Both chapters describe experiments in climate-controlled aviaries where breeding pairs of great tits were kept under a natural photoperiod, but varying temperature treatments. The experimental design followed from an experiment described by Visser et al. (2009) that used a similar setup and in which birds were exposed to two temperature profiles, mimicking the temperatures from a year in which mean laying dates of wild great tits were either very early or very late. Using this setup, Visser and colleagues could show that laying dates in captive birds were indeed directly affected by temperature, when influences of other phenological stimuli were ruled out. The four experiments described in **chapters 3** and **4**, performed in 2006 to 2010, were set up to look more specifically at which component of this complex temperature profile did affect egg laying dates. **Chapter 3** describes an experiment in which pairs of great tits were exposed to two temperature treatments that both showed a similar seasonal temperature increase, but differed by 4°C in mean temperature. In contrast to Visser et al. (2009), this temperature setup did not affect laying dates. In **chapter 4** we present the outcome of three experiments in union. The temperature treatments the birds were exposed to varied in the timing of a temperature increase, the daily temperature variation and the timing, steepness and mean temperature of a seasonal temperature increases. In combination, these five experiments demonstrated clearly that the pattern of temperature increase, rather than mean temperature *per se* is used as a predictive cue by female great tits to time their onset of egg laying. In addition, we could demonstrate that related individuals resemble each other in their cue sensitivity, pointing towards a genetic basis, which could allow a response to selection, i.e. an adaptive change. We could also show that even though temperature developments well-ahead of the actual laying event did influence the timing of laying, the underlying reproductive physiology was not affected by temperature changes. This opens up the question of how temperature cues are transduced into a laying decision, if they are not affecting gonadal growth itself.

In **chapter 5** we more closely investigate the individual variation in the timing of endocrine changes and gonadal maturation and their relevance for the decision

when to lay. This comparison has further relevance as our dataset of laying dates in captivity under controlled conditions combined with measures of reproductive physiology is almost unique and allows an evaluation of the relevance of using physiological proxies as an indicator for the timing of reproduction itself. We show that besides consistent individual variation in the timing of reproductive maturation, increases in reproductive hormones, gonadal growth and the onset of laying are at best weakly correlated with each other. This demonstrates that the responsiveness to environmental cues might change over developmental stages, or different stages might be responsive to different cues. Our results question the relevance of studies using the response of physiological proxies to environmental cues as an indication of the influence of these cues on avian breeding times.

As we are interested in possible population-level changes in reproductive timing in response to increasing spring temperatures, in **chapter 6** we further investigated the heritability of the extent of gonadal maturation. Even though we demonstrated a female's potential to fine-tune her laying decision in response to temperature cues independent of the stage of gonadal maturation in the current situation, a further advancement of egg laying, as necessitated by climate warming, could indeed require an advancement of gonadal growth itself. By comparing gonadal growth within siblings we demonstrate high heritability in the photoinduced extent of ovarian follicle maturation in early spring. Furthermore, we show how the heritable component explaining the variation decreases over time and interpret this divergence between siblings as different growth rates in response to the encountered environment.

Chapter 7 investigates the effects of ambient temperature on egg size. Causes and consequences of egg size variation remain still poorly understood, but from observational studies it is known that egg size increases seasonally with rising temperatures and a higher food abundance. In this chapter we investigate egg size variation of great tits kept in climate-controlled aviaries exposed to *ad libitum* food and a wide range of experimental temperatures and compared this variation to the variation found in the wild source population. We demonstrate that egg size was far more variable in birds that were not food-restricted than under natural conditions, especially between females, but less so if these were sisters. In contrast to the wild population, egg size did not increase with temperature. We thus conclude that egg size variation in nature is mainly driven by the dynamics in food abundance.

In **chapter 8** we come back to the principal question of how variation in temperature can affect seasonal timing and discuss correlational, but also causal evidence for direct temperature effects on reproductive timing in birds and mammals. Furthermore, we take a more mechanistic approach and explain possible modes of temperature perception and integration, a subject that still brings forward more questions than answers and develop a roadmap to the future.

In the two following chapters, we investigate effects of non-temperature cues on reproductive timing in great tits. In **chapter 9** we demonstrate that the exposure to just one single long day in winter induces subsequent gonadal growth in captive female great tits. In contrast, in wild birds that were exposed to the same treatment in spring, the photoperiodic stimulus did not lead to an advancement in laying date. This shows once again the overriding effect of supplementary cues, such as temperature, on the final laying decision.

In **chapter 10** we first review current evidence for an association between phenological and climatic cues and the onset of reproduction in opportunistically breeding species and strictly seasonal birds, like the great tit. To directly test a causal relationship, we performed an experiment investigating the effect of phenological cues on the timing of hormonal changes and egg laying in captive great tits. Even though we could demonstrate a direct effect of temperatures on the decision of when to lay, we could not exclude that this effect could be intensified by cues from the environment that are closely associated with temperature rises and can be seen as an integration of past temperature developments. Likely candidates for predictive cues are temperature-sensitive trees, like birch, or the seasonal increase in food abundance. After establishing a correlation between the bud burst of birches and the laying dates of great tits in the wild study population, we exposed captive breeding pairs housed in open outdoor aviaries under natural photoperiod and climate to sprouting birch branches and lepidoptera larvae. That these supplementary cues neither affected the early-spring increase in reproductive hormones nor the onset of laying again emphasises the importance of temperature cues in timing the onset of reproduction.

In **chapter 11** we consider if small-scale variation in mean temperature around the nest boxes that wild great tits bred in affects the onset of laying of individual birds, thus partly causing the observed variation between early- and late- laying females under natural climatic conditions. We placed a grid of temperature loggers over the Hoge Veluve study area and could thus associate ambient temperatures close to an individual nest box with the timing of reproduction of great tit females breeding in these boxes. In contrast to the negative correlation between mean temperature and the population mean onset of laying across years, individual females breeding in warmer habitats showed a slightly delayed laying date. However, this effect clearly cannot account for the large variation in first laying dates observed in the population, therefore suggesting different mechanisms, such as variation in the sensitivity to temperature cues or condition-related costs as causes for between-female differences in the onset of reproduction.

Finally, I summarize and discuss the main findings in a synthesis and propose future research directions in **chapter 12**.

Chapter 2

Marcel E. Visser, Samuel P. Caro , Kees van Oers, Sonja V. Schaper and Barbara Helm

Phenology, seasonal timing and circannual rhythms: towards a unified framework

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Abstract

Phenology refers to the periodic appearance of life-cycle events and currently receives abundant attention as the effects of global change on phenology are so apparent. Phenology as a discipline observes these events and relates their annual variation to variation in climate. But phenology is also studied in other disciplines, each with their own perspective. Evolutionary ecologists study variation in seasonal timing and its fitness consequences, whereas chronobiologists emphasize the periodic nature of life-cycle stages and their underlying timing programmes (e.g. circannual rhythms). The (neuro-) endocrine processes underlying these life-cycle events are studied by physiologists and need to be linked to genes that are explored by molecular geneticists. In order to fully understand variation in phenology, we need to integrate these different perspectives, in particular by combining evolutionary and mechanistic approaches. We use avian research to characterize different perspectives and to highlight integration that has already been achieved. Building on this work, we outline a route towards uniting the different disciplines in a single framework, which may be used to better understand and, more importantly, to forecast climate change impacts on phenology.

1. Introduction

Phenological records of periodically recurring lifecycle events go back thousands of years (Foster & Kreitzman 2009). One of the striking observations is the large difference in the between-year variation in phenology: some seasonal events occur so reliably, for example the return of certain migratory species, that they have been likened to 'calendars', whereas others show high year-to-year variation in the date at which they occur (Gwinner & Helm 2003). In the temperate zone, annual variation in phenology often correlates with environmental variables, most often with temperature. It is therefore not surprising that global climate change, including an increase in average temperatures, has led to clear shifts in phenology, but also with large differences between species (Schwartz 2003; Parmesan 2006). The variation in response to climate change between species at different trophic levels indicates that many phenological shifts currently remain inadequate and lead, for example, to uncoupling of phenological events within food chains (Visser et al. 1998; Visser & Both 2005; Memmott et al. 2007; Post & Forchhammer 2008; Miller-Rushing et al. 2010; Singer & Parmesan 2010). Mistiming has consequences at the population level (Nussey et al. 2005; Both et al. 2006). Thus, phenology is a key process that may link climate change to population persistence and possibly to community composition (Miller-Rushing et al. 2010).

In order to assess the ecological consequences of climate change it is essential to forecast phenology under different scenarios, such as provided by the Intergovernmental Panel for Climate Change (IPCC). This forecasting is hampered

by two major problems (Visser 2008): phenology needs to be forecasted for environments well outside the range of natural conditions observed by phenologists. Predictions must therefore rely on additional information, in particular about the causal (mechanistic) basis of the relationship between phenology and environmental conditions. Furthermore, organisms may adapt via micro-evolution. Hence, the relationship between phenology and environment is changing over time, and this rate of adaptation needs to be incorporated in the forecasting (van Asch et al. 2007). To meet these two challenges, it is crucial that research on phenological events integrates mechanistic and evolutionary perspectives.

A variety of disciplines study the seasonality of plant and animal life-cycle events. These include researchers who consider themselves phenologists but also ecologists, who would term it seasonal timing, and chronobiologists, who focus on timing programmes and on underlying mechanisms such as circannual rhythms. Similarly, physiologists, who are studying reproduction or any other seasonal life-cycle stages, and more recently molecular ecologists who look at the genetic make up of individuals, are also interested in within-season variation (e.g. Wilczek et al. 2010). It is obvious that while all these disciplines deal with the same phenomenon, they take different angles to it and aim at different endpoints. This is due to historical differences, as they approach phenology after having developed in diverse contexts. Over recent decades, all disciplines have made remarkable progress in unravelling detailed information underlying phenology and although there have been a number of excellent examples of integration (see §3), we believe this is the time to promote further integration of different disciplines.

In this paper, we will highlight the insights and the limitations of the diverse approaches to phenology, and we outline a route towards uniting the different disciplines in a single framework. We will use this framework to provide an outlook to what kind of research is needed to forecast phenology influenced by climate change. As the common basis of our combined backgrounds, we use avian timing as an example of how integration could be achieved.

2. Different perspectives

Different approaches to phenology focus on different aspects of seasonal phenomena. Researchers in the field of phenology observe in a standardized way periodic plant and animal life-cycle events over long periods of time, sometimes using phenological stations (Menzel & Fabian 1999; Schwartz 2003) and relate the inter-annual variations to climatic variables. Thus, they approach seasonal recurrence as a phenomenon in its own right, with a clear interest in year-to-year variation, but mainly in the first individuals or (less often) the population mean rather than the variation among all individuals of a population. Phenologists are well aware of (climate) changes in the

long run and can provide a comprehensive picture of modified timing on a level of local populations and communities, often in a wide range of species and over large geographical regions.

In contrast, population-level data are less central for other disciplines studying phenology. Phenological events, such as flowering date, return date of birds and egg laying date of sea turtles, can also be studied as characteristics of individuals within populations. In this approach, the phenological event is seen as the phenotype of a given individual. As a step towards integrating disciplines, studies of phenological trait values as phenotypes can be framed in the concept of phenotypic plasticity (Pigliucci 2005). If an individual is phenotypically plastic, its phenotype is shaped by the interaction between the genotype and the environment. Different genotypes in the same environment will give rise to different phenotypes, while the same genotype will lead to different phenotypes in different environments (Fig. 2.1a). The curve describing the relationship between phenotype and environmental variables is termed the reaction norm. While traditionally phenotypic plasticity was used for morphological traits shaped during ontogeny, it is now also widely used for traits that are expressed multiple times in an organism's life (Nussey et al. 2005), such as phenological events. An example would be the lay date of an individual bird in different spring environments over consecutive years.

Below, we use the conceptual background of phenotypic plasticity to highlight differences between disciplines and to characterize their particular approaches to explain a specific phenological event in a given year, focusing on avian reproduction.

(a) The evolutionary ecologist's view

Evolutionary ecologists refer to phenological events as seasonal timing and put emphasis on the variation among individuals within years as well as on the between-year variation. Year-to-year variation should ultimately be explained by corresponding year-to-year variation in the seasonality of the environment (Baker 1938; Visser et al. 2004). For example, timing of reproduction in small forest passerine birds of the temperate zone is affected by temperature because the time of the peak abundance of the nestlings' food is correlated with this temperature (Visser et al. 2006). Well-timed breeding is thought to confer benefits from higher fitness both in terms of enhanced offspring survival and possibly increased condition of the parents to survive until the next breeding season (Thomas et al. 2001; Fig. 2.1b). Thus, in an evolutionary ecologist's view, birds have been selected for their ability to have their chicks in the nest at the time of peak food abundance.

However, timely reproduction requires anticipation of suitable conditions well in advance. A bird has to make a 'decision' whether to initiate preparations for breeding long before its chicks will be exposed to the environmental conditions that determine

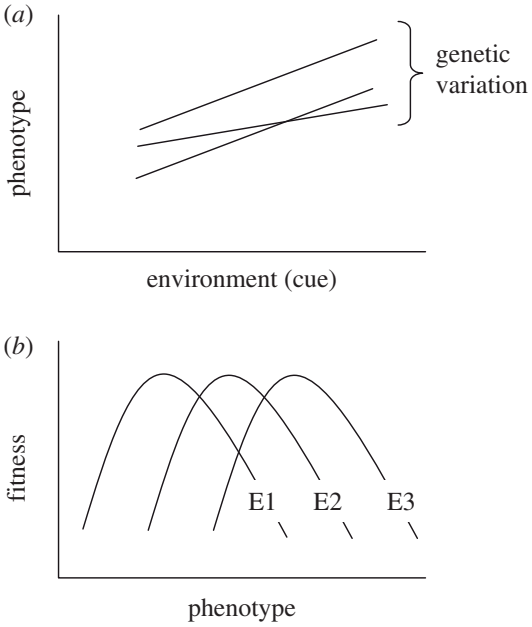

Figure 2.1: (a) A phenological trait value, the phenotype, can be shaped by the environment: the same genotype gives rise to different phenotypes in different environments (the trait is phenotypically plastic). Different genotypes have different reaction norms: their phenotypes are affected differently by the environment; environmental factors on the x-axis represent those that are used as predictive cues for phenology. (b) Different phenotypes have different fitness depending on the environment (E1–E3). Note that the environment in (a) is often a different environment than the environment of selection in (b) (see text).

reproductive success. Thus, environmental variables at the time of 'decision-making' are often used as predictive cues and thereby also function as proximate (mechanistic) causes that influence reproductive timing (Baker 1938) Because the use of cues is crucial for optimal timing, an evolutionary ecologist wants to know whether responsiveness to environmental information (cues) has evolved to correctly predict the time to initiate breeding. An evolutionary ecologist is not primarily interested in the causal mechanism but is very much aware what the cues should provide: they should predict the future environment under which the phenotype will be selected. Key characteristics of this future environment of selection include conspecific and multi-trophic interactions. Thus, evolutionary ecologists try to find the cues that are reliably linked to these characteristics. There is no *a priori* reason why just a single environmental variable should act as a cue, and it is a pragmatic oversimplification that evolutionary ecologists often consider phenotypic plasticity as the relationship between timing and just a single environmental variable. More generally, the reaction norm should have a multi-dimensional environmental axis (Visser 2008).

Evolutionary ecologists mainly study organisms in the wild as they are interested in the fitness benefits of different timing strategies and in selection on timing. In collaboration with quantitative geneticists they estimate the heritability of timing and the response to selection; i.e. the rate of micro-evolution. An important limitation is that extrapolation beyond the natural range, as needed for predictions under climate change, is not feasible without a more mechanistic understanding of phenology (see also §4a).

(b) The physiologist's view

Physiologists have a clear interest in how the changes within an annual cycle causally come about. They ask how organisms use environmental cues to adjust changes in morphology, physiology and behaviour over the course of their annual schedule, often with a strong emphasis on the effects of photoperiod (the annual change in day length) on the orchestration of successive life-history stages (i.e. progression of events; Farner et al. 1966; Follett et al. 1985; Wingfield 2008a). The physiological mechanisms underlying avian timing of reproduction, one of the most explicit examples of phenology, describe how cues are perceived and transduced at the level of the hypothalamo–pituitary–gonadal (HPG) axis (Fig. 2.2). These environmental cues have been classified in several different groups (Wingfield & Kenagy 1991). The most important ones for seasonal breeding are probably the initial predictive cues, which allow preparations well ahead of breeding (i.e. photoperiod), and the supplementary and social cues (i.e. temperature, rainfall, interactions with conspecifics, food abundance), which allow fine-tuning of timing to local, and year-specific, conditions.

Photoperiodism has been massively studied and the effects of photoperiod on the HPG axis are well described (Fig. 2.2; see reviews in Farner 1985; Follett et al. 1985; Dawson et al. 2001; Sharp 2005), while our knowledge of the effects of the supplementary and social cues is patchy. Most insights have been achieved on the effect of temperature as a cue (Wada et al. 1990; Silverin & Viebke 1994; Wingfield et al. 1996; Wingfield et al. 1997; Maney et al. 1999; Meijer et al. 1999; Wingfield et al. 2003; Perfito et al. 2005; Salvante et al. 2007; Silverin et al. 2008; Visser et al. 2009) but there is a serious lack of understanding on how temperature is integrated at the level of the HPG axis (see §4b). Similarly, some studies have shown marked effects of other supplemental cues, such as food and water availability and social cues (Moore 1983; Vleck & Priedkalns 1985; Hahn et al. 1995; Zann et al. 1995; Hau et al. 2000; O'Brien & Hau 2005; Helm et al. 2006; Small et al. 2007; Voigt et al. 2007; Perfito et al. 2008). Most of these studies were carried out on a behavioural level, but some are now addressing links to the HPG axis (e.g. effects of social cues; Moore 1983; Stevenson et al. 2008).

Physiologists are in general less interested in year-to-year variation or variation among individuals (Ball & Balthazart 2008; Williams 2008; but see Wingfield et al. 1992) than in detailed, typically experimental, studies of individuals. In an environmental context, physiologists study between-species (or between-populations) variation within an annual cycle with an emphasis on average values. Classically, physiology often requires careful measurements under controlled conditions, and hence much of the work is done in the laboratory and restricted to a specific mechanism. This has major advantages and physiologists have made important contributions to our knowledge of the detailed molecular and genetic basis of the physiological system. When related to phenology of free-living animals there are, however, a few

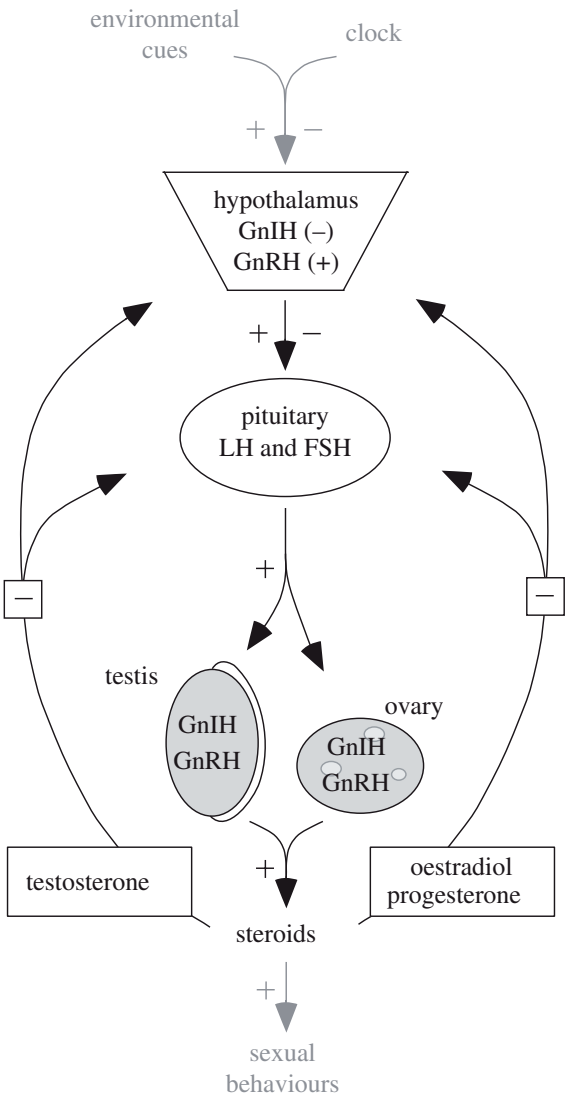

Figure 2.2: A classical view of the hypothalamo–pituitary–gonadal (HPG) axis (in black) and its integration with phenology (in grey): the relevant environmental cues (e.g. photoperiod) interact with the permissive clock mechanisms to stimulate (plus sign) or inhibit (minus sign) the secretion of the gonadotropin-inhibitory (GnIH) and -releasing hormones (GnRH) by the hypothalamus into the portal veins, which in turn interact to regulate the release of the gonadotropins (luteinizing hormone, LH, and follicle-stimulating hormone, FSH) by the pituitary into the general circulation. LH and FSH bind to receptors in the ovary and testis, stimulate their development, the gametogenesis and their production of steroid hormones (mainly testosterone in males, oestradiol and progesterone in females). These steroids are involved in other physiological and morphological changes (e.g. secondary sexual characters) and increase the probability of several sexual behaviours, such as courtship and egg-laying, occurring. They also act through negative feedback mechanisms on the higher levels of the HPG axis. Note that GnIH and GnRH have recently been identified in the gonads as well, where they potentially act as regulators (see review in Ubuka et al. 2008).

shortcomings (Calisi & Bentley 2009). For example, the environmental variables used in an experimental set-up usually are set to arbitrarily chosen values (often well outside the natural range) and often these variables are kept constant under experimental conditions (e.g. 20°C throughout the day and night, 16 h of light for several consecutive weeks).

A disadvantage specific for work on phenology of avian reproduction is the use of male rather than female animals (Ball & Ketterson 2008) while probably females are more important in determining seasonal timing (Caro et al. 2009). Partly this is because under captive conditions males commonly develop fully active reproductive conditions, while females often do not reach full breeding status, especially when caged individually (but see Calisi & Bentley 2009). As a consequence most studies do not describe the links between physiological mechanisms and the complex interactions that determine lay dates in the wild. Physiological studies commonly study gonadal (generally testis) development to examine reproductive cycles. This allows a detailed understanding of different phases in the breeding cycle and of their respective regulatory mechanisms. However, the value of gonadal cycles as proxies of lay dates may be limited, and there have been few attempts to validate these proxies against actual field data (Helm 2009; Visser et al. 2009). Gonadal development is, however, also studied by physiologists as a seasonal process in itself, rather than as a proxy for actual laying dates.

(c) The chronobiologist's view

Chronobiologists study daily and annual fluctuations in physiology and behaviour and focus on internal timing programmes that enable organisms to cope with, and anticipate, geophysical cycles in the environment. Fundamental for this field is the observation that periodic events on a daily or annual scale often persist endogenously, i.e. in complete absence of external time information given by *Zeitgebers* (timing cues) like light and darkness. Such endogenous daily ('circadian') or annual ('circannual') rhythms continue with period lengths that differ slightly from 24 h or 365 days, respectively (Gwinner 1986; Gwinner 2003; Kumar et al. 2004; Bradshaw & Holzapfel 2007; Paul et al. 2008; Helm 2009). Thus, without any seasonal cues, many animals can maintain annual cycles of moult, migration, gonadal development, pupation or hibernation for many years (Gwinner 1986; Nisimura & Numata 2001; Kondo et al. 2006) by solely relying on changes of their circannual clock. *Zeitgebers* synchronize rhythms by determining their period (i.e. the length of a cycle) and their phase (i.e. the time when a particular fraction of the cycle occurs), and additionally, other factors may modify ('mask') the expression of rhythms. However, species differ greatly in the strength of the underlying circannual clock and in requirements for the environmental input (Dawson et al. 2001; Goldman et al. 2004; Bradshaw & Holzapfel 2007; Paul et al. 2008; Helm 2009).

When chronobiologists look at phenology, they focus on the annual cycle and variation over a year, but are less interested in variation between years and individuals. The chronobiologist's view on why phenological events occur and when they occur emphasizes the interplay between internal time-structuring and environmental cues. The seasonal clock determines how sensitive an animal is to external cues (Helm et al.

2009). These cues will for some phases of the seasonal clock lead to stimulation of the system, while at other phases they will have little or no impact (see below).

The chain of steps from cue input to the specific output, i.e. laying date, is still poorly understood, but it involves a calendar-and-clock system. The much better known circadian clock entrains to the 24 h light cycle and provides a 'reference clock' for the reading of calendrical information (i.e. photoperiod; Sharp 2005). The calendrical information that is thereby attained is modulated by a bird's internal (circannual) calendar, e.g. interpreting a 12 h day as 'long' or 'short', respectively in a seasonal context (Helm et al. 2009). If a bird is in the correct phase for photostimulation, a cascade of gene expression starts, and the genes involved in this cascade are now being rapidly revealed (Ono et al. 2009). Subsequently, in different brain regions, different aspects of physiology and behaviour are activated that further prepare reproduction (e.g. growth of song nuclei, melatonin receptor density change; Bentley & Ball 2000). The activation and development of these processes then leads, via physiological and behavioural feedback loops, to specific responsiveness to relevant environmental factors (nutritional levels, social stimuli, etc.). Thereby, the interplay between clock and local environment determines the precise timing of the phenological event, i.e. the seasonal phenotype.

A limitation of this approach is a focus on experimental, and often constant, conditions (Calisi & Bentley 2009). Thus, despite the stated interest of chronobiologists in understanding temporal behaviour, relatively little is known about how the underlying calendar-and-clock system relates to 'timing in the real world' (Menaker 2006; Wikelski et al. 2008). However, comparative studies of related taxa indicate that timing programmes are tailored to particular life histories, and an evolutionary angle is starting to develop (Gwinner 1986; Bradshaw & Holzapfel 2007; Helm et al. 2009).

(d) The molecular geneticist's view

Molecular geneticists approach phenological events from two sides: on one hand, they aim at understanding the genetic variation among individuals in the timing of the event and the selection on this variation. On the other hand, they aim at understanding the causal mechanism underlying the effect of the environment on the event. Several approaches can be used to identify genes that are involved in the variation among individuals (the genotypes for phenological events; Tauber & Kyriacou 2005). The polymorphisms can be found either by a candidate gene approach, where orthologues of (mainly mammalian) clock genes are cloned (candidate gene approach; e.g. Chong et al. 2000; Yoshimura et al. 2000; Fidler & Gwinner 2003), or by a genome-wide approach, where random markers are used to identify the variation in genome regions (polymorphisms) that are associated with the between-individual variation in a trait like the timing of a phenological event (quantitative trait locus (QTL) approach; e.g. Leder et al. 2006).

In birds, only one candidate gene has been investigated in an ecological or evolutionary seasonal context: *clock*. Repeat length variations are reported to vary between species (Fidler & Gwinner 2003), between populations (Johnsen et al. 2007) and within natural populations (Liedvogel et al. 2009). Johnsen et al. (2007) showed that in blue tits, a latitudinal cline exists in mean repeat length, with a higher mean repeat number at higher latitudes. They hypothesize that these population differences may be caused by adaptation to the variation in photoperiodic parameters between the populations (Johnsen et al. 2007). This was tested on a within-population level by Liedvogel et al. (2009), who found that females with shorter mean repeat lengths had earlier lay dates. Whereas ecological studies using candidate genes are rare, to the best of our knowledge, studies that investigate the adaptive significance of gene expression profiles are completely absent in wild birds.

Investigation of genes involved in a causal pathway is done by exploring the variation in transcription, translation and post-translational expression level (through e.g. mRNA, microarrays, proteomics) of clock genes. The expression of these genes has been localized in several brain areas, including the avian pineal gland, putative suprachiasmatic nuclei (SCN), retina and hypothalamus (Yasuo et al. 2003; Kumar et al. 2004). The expression levels give insight into the physiological processes that are up- or downregulated, causing rhythmic phenotypic expression of a trait. For this purpose, most genetic information on seasonal timing comes from research done on circadian rhythms.

The two molecular genetic approaches to phenological events (i.e. a focus on genomic variation and a focus on expression of causal pathways) are likely to identify common, but also unique sets of genes involved in regulation of heritable quantitative traits (Le Mignon et al. 2009). For instance, there may be no genomic variation in some of the genes that play a crucial role in the pathway. These can therefore not account for genetic variation among individuals and thus cannot be picked up using a QTL approach (e.g. Zou & Zeng 2009). However, they may be identified in expression studies, since the expression levels of these specific genes may be important drivers of a rhythmic expression of a trait and may be heritable by themselves (Brem & Kruglyak 2005). The differences between the approaches can be illustrated by comparison to an engine that can be set by a switch and is thereafter driven by a converter belt. Based on this analogy, microarray studies are likely to pick the genes involved in the 'converter belt', i.e. those genes that are involved in rhythmic seasonal fluctuations in reproductive hormones. On the other hand, QTL studies may be more likely to pick up the 'switch genes' that set the converter belt in motion. Physiologists, in collaboration with molecular geneticists, therefore play an important role in the investigation of both the expression of 'converter belt' genes and 'switch genes' (Ono et al. 2009).

A limitation in molecular genetic research on seasonal timing, and on phenological events in general, is that the genetic basis that underlies circannual rhythms is

unknown. While day-length-dependent gene expression is relatively well understood, the knowledge of genes that underlie rhythmic expression over the season still needs to be developed. Another limitation is that conflicting results often arise owing to the use of different species, different photoperiods, variation in entrainment protocols and potentially different mRNA detection techniques (Helfer et al. 2006). This makes a comparative study between species with different life histories problematic.

3. Integration towards a single framework

The approaches to phenology outlined above have the potential to complement each other. In Fig. 2.3, we outline a common framework that refers to all these disciplines, with the aim of a more conclusive understanding of phenology. Below, we develop this framework in further detail and emphasize promising steps towards integrating the different approaches. A first section gives a combined overview over the sequence of processes leading to a phenotypic event (in our example this is timing of avian reproduction). A second section briefly introduces a model system of integration, reproductive timing in blue tits (*Cyanistes caeruleus*).

(a) An integrated view of a phenological event

The trait that is observed in phenological studies (the phenotype) will be under natural selection, and hence, the processes that are studied by all above disciplines occur in an evolutionary context. Often, relatively small differences in timing of reproduction or arrival date in migrant bird species have large fitness consequences (Nussey et al. 2005; Pulido 2007). Because fitness consequences depend on a species' ecology and the particular environment, there is no single mechanism that fits all species. Thus, a crucial step towards understanding a given phenological event involves taking account of variation between species or populations, as well as between individuals within a population.

(i) Shaping the phenotype

An animal perceives cues from its environment. Cues are environmental variables that have predictive qualities for subsequent, suitable conditions to carry out a given life-cycle event. The perception of suitable cues will affect an animal's decision to initiate preparatory steps, and hence, affects phenology (seasonal timing). Cues can be abiotic factors, like temperature, but also biotic factors, like the development of the vegetation or social cues from conspecifics. Which environmental variables actually are cues varies from species to species and sometimes from population to population within a species. The cues used by an animal need to predict the environment of selection, i.e. the conditions under which the selection on the phenological trait takes place, in order to be useful. For example, rainfall does not predict the phenology of

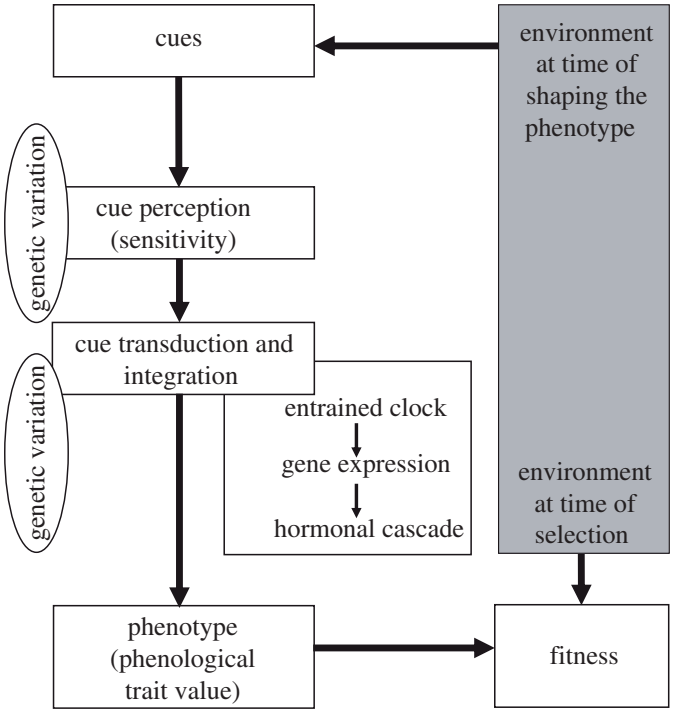

Figure 2.3: A unified framework for a more conclusive understanding of phenology, integrating chronobiology, physiology, molecular genetics and evolutionary ecology (see §3 of the text).

forest caterpillars and is thus not a cue for a great tit (*Parus major*) in the Netherlands, but rainfall does predict the phenology of ripe grass seeds and it is therefore a cue for zebra finches (*Taeniopygia guttata*) in Australia (Zann et al. 1995; Perfito et al. 2006). This kind of variation even exists within species. Different subspecies of white-crowned sparrows (*Zonotrichia leucophrys*) differ in whether or not they use temperature as a cue; the Arctic subspecies shows no response to temperature in their testis and follicle size development and this can be understood from the fact that the short Arctic breeding season is restricted to a period of long photoperiod, while other subspecies have to rely on supplementary cues to account for between-year variation (Wingfield et al. 1996; Wingfield et al. 1997; Maney et al. 1999; Wingfield et al. 2003). A similar pattern has been found for great tits (Silverin & Viebke 1994; Silverin et al. 2008).

The animal's sensitivity to cues varies over the year as the response mechanism is seasonal clock-dependent. Photoperiod is generally seen as the most powerful seasonal cue. Many organisms, including birds, can be induced to carry out phenological events 'out of season' by photoperiodic manipulation (Dawson et al. 2001; Goldman et al. 2004; Bradshaw & Holzapfel 2007), and some can be forced to undergo several annual cycles within a single year (Gwinner 1986). But even responses to photoperiod depend on the phase of the underlying, circannual cycle (Miyazaki et al. 2005; Lincoln et al. 2006; Helm et al. 2009). For example, while increasing day length stimulates breeding early in the year, even permanent light cannot re-stimulate reproduction after breeding in most species ('photorefractoriness'; Dawson et al. 2001; Hahn & MacDougall-Shackleton 2008). In addition, there is also evidence for possible effects of temperature on circannual clocks (e.g. in hibernating mammals; Mrosovsky 1986) and for dependence of the sensitivity to temperature on the phase of the clock. For example, temperature does not influence the physiological breeding development in quails (*Coturnix coturnix japonica*) but a decrease in temperature, in combination with a decrease in photoperiod, is necessary to fully stop their reproductive activity (Wada et al. 1990; Wada 1993). In other species, the same temperature has a stronger effect under longer photoperiods (i.e. later in spring) than under short photoperiods (Gienapp et al. 2006; Bauer et al. 2008) and early versus late during mammalian hibernation (Wikelski et al. 2008). Similarly, when the photoperiod gets very long, it can override the lack of supplemental cues and birds can breed under very low ambient temperatures (e.g. Lambrechts et al. 1997b).

Cues from the environment are thus perceived, transduced and integrated by an animal's clockdependent and -independent response mechanism. This leads to genes being switched on (initially in the brain), which in turn may switch on the genes involved in the pathway processes (the 'converter belt' genes). After these converter belt genes have been activated, a complex process with positive and negative feedback loops is started. Hormones clearly play a crucial role in this process and some pathways are well understood. The perceived environmental cues induce a cascade of neuroendocrine reactions along the HPG axis that are essential in the synchronization of the breeding cycle (Fig. 2.2). These hormones increase the probability of sexual behaviours occurring, but also play various morphological and physiological roles, including some negative feedback on higher levels of the HPG axis (Wingfield & Moore 1987; Ball & Balthazart 2002; Dawson & Sharp 2007).

While the successive levels of the HPG axis and their interactions have been well described, our knowledge on how supplementary environmental cues, other than photoperiod, are modulating the maturation of the reproductive system remains rudimentary. This is particularly true for the late stages of reproductive development in females, the rapid follicular growth that occurs in the last days before egg laying. As a consequence, the link between the activation and modulation of the HPG axis and

the actual egg laying is less well understood. Under cold conditions, egg laying will be delayed in the field and perhaps temperature plays a role in this final fine-tuning stage (Meijer et al. 1999). But also unpredictable events such as severe weather conditions or territory loss will determine the laying decision and may induce temporary switching to an emergency life-history stage (Wingfield et al. 1998).

(ii) Selection on the phenotype

Animals within populations are genetically diverse. One can think of genetic variation in cue perception, transduction and integration. This genetic variation will lead to differences in the reaction norm (Fig. 2.1a). In some cases, individuals will vary in how much their phenotype is responsive to variation in cues, which will lead to variation in the slope of the reaction norm (Nussey et al. 2005). In addition, some animals will have a consistently earlier phenotype than others, and these will differ in the elevation of the reaction norm.

Teasing apart genetic variation at the perception level from genetic variation in the transduction mechanisms is a difficult task, especially in a phenological context where the cues acting on the physiology are gradually interacting and have long-lasting effects. This challenge can only be addressed if brain regions responsible for the filtering and the processing of environmental information are known. Researchers can then determine whether the expression of genetic variation occurs before or during the transduction of the signal considered as a relevant cue. In the context of seasonal reproduction, the pioneering studies by Heideman and colleagues on white-footed mice (*Peromyscus leucopus*; e.g. Heideman et al. 1999; Heideman & Pittman 2009) are important steps in this direction. Populations of white-footed mice differ geographically in the extent to which reproduction is inhibited under short day lengths (Heideman et al. 1999). By selective breeding and quantitative genetic analyses of variation, Heideman and coworkers showed high heritability of photoresponsiveness. Follow-up studies tracked down heritability of gonadotropin-releasing hormone (GnRH) neuron characteristics and also provided evidence for genetic variation in the nutritional and hormonal inputs to GnRH neurons (Heideman & Pittman 2009). Limited evidence exists also for plasticity and genetic variation in the perceptual mechanisms. For example, seasonal and population variations in hearing capabilities have, respectively, been demonstrated in several species of birds (Lucas et al. 2002; Lucas et al. 2007) and in at least one species of frog (the cricket frog, *Acris crepitans*; Ryan et al. 1992).

Regardless of whether genetic variation affects the perception or the transduction level, variation in the way cues are 'translated' into a phenotype enables natural selection to act on phenotypic plasticity; i.e. on the reaction norms (Visser et al. 2004; Heideman & Pittman 2009). Thus, it is not so much the trait value itself that selection

acts on but rather the physiological response mechanism underlying the phenotype. This also implies that perhaps not all the reaction norms are possible, as evolution may be constrained by the particular components of the response mechanism that show genetic variation. Furthermore, natural selection probably rarely acts on isolated physiological mechanisms, but rather on a suite of correlated traits (McGlothlin & Ketterson 2008). This is particularly true for hormones that often act simultaneously on a wide variety of characters (Ketterson & Nolan 1999), which may also constrain the response to natural selection (Lessells 2008, but see Hau 2007). Similar constraints could apply to modifications of the circadian clock system that is involved in seasonal as well as daily timing (Bradshaw & Holzapfel 2007; Heideman & Pittman 2009).

How well a phenotype performs in terms of fitness depends on the environment. For many species, the phenology of their environment, and thus of their food sources or their predators, varies from year to year. The fitness of a bird making a decision on a certain date therefore varies from year to year. It is thus a combination of the environment at the time of selection and the phenotype that determines the fitness of an individual (Fig. 2.1b).

In many cases the environmental variables that serve as cues, involved in shaping the phenotype, are not the same environmental variables that form the environment at the time of selection (Visser et al. 2004). Especially in phenology, the phenotype is often formed in a different environment from the one where selection takes place. For example, in migratory birds, the decision to depart the wintering grounds is made at a different location (environment) than the place where they are selected to be on time (their breeding grounds). This is also depicted in Fig. 2.1a,b: in Fig. 2.1a the phenotype is determined by responses to predictive environmental cues (x-axis) while in Fig. 2.1b the fitness of a given phenotype depends on the environment of selection (E1–E3). This highlights the importance of a correlation between the 'cue' and the 'environment of selection': only if the environment of selection is predicted by an environmental variable can it serve as a predictive cue.

(b) An example of interdisciplinary integration: reproductive timing in Mediterranean blue tits

In the Mediterranean region, bird populations have to cope with strong habitat heterogeneity owing to geographical variation in the vegetation structure of the landscape. Mediterranean forests consist of a mosaic of patches dominated either by broad-leaved deciduous or evergreen tree species. Blue tits successfully breed in both habitats, but have to deal with pronounced spatial variation in the phenology of their food. Deciduous forests present an early phenology, with young leaves appearing approximately one month earlier than in evergreen woods. This temporal difference in bud burst results in a similar difference in the onset in leaf-eating caterpillar

outbreaks between the habitats (Zandt et al. 1990). Blue tits, like many other insectivorous bird species, depend highly on that brief peak in caterpillar abundance to raise their chicks. Differences in the timing of food abundance have important consequences for fitness and have been the subject of considerable research in the field of evolutionary ecology over the past 30 years (Blondel et al. 1993; Lambrechts et al. 1997a; Blondel et al. 1999; Lambrechts et al. 2004; Blondel et al. 2006). In fact, the geographical and temporal variability in the availability of this food resource is one of the main selection factors driving the breeding phenology of the different blue tit populations, with high energetic and survival costs for birds failing to match their chick-rearing period with the annual short peak in caterpillar availability (Thomas et al. 2001). Since the discovery that blue tits may breed up to one month apart depending on the type of habitat in which they settle (Blondel & Isenmann 1979), these birds have been used in many studies aiming to understand how the phenology of breeding has evolved. Furthermore, research examined how this local adaptation has sometimes been constrained by the characteristics of the habitats and the birds' ability to adaptively respond to selection processes (e.g. the homogenizing effect of gene flow; see Dias 1996; Blondel et al. 2006).

Selection operates on the mechanisms underlying phenotypic plasticity and local specialization rather than on the phenotype itself (see above), and thus a significant part of the work conducted on these blue tit populations aimed to decipher the proximate organization of their breeding phenology. Furthermore, as studies of the proximate organization mainly aimed at mechanistic responses to environmental cues, a combination of field data and common garden experiments manipulating these cues was necessary (Visser & Lambrechts 1999). So, the breeding phenology of blue tits from different Mediterranean populations was also studied in captivity, first in outdoor aviaries exposed to natural environmental conditions, then under diverse combinations of natural and artificial photoperiods. Under natural settings, the laying divergence observed in the field persisted, suggesting an (at least partial) genetic determination of these population differentiations (Lambrechts & Dias 1993). Under manipulated day lengths, the breeding difference was modulated by the duration of the photoperiod, suggesting that the laying differentiation was maintained by a population divergence in the perception or transduction mechanisms to photoperiod (Lambrechts et al. 1996; Lambrechts et al. 1997b; Lambrechts & Perret 2000). Subsequent work on Corsican birds combined behavioural, neurobiological and endocrinological studies in the field and sought to describe where in the HPG axis the population differentiation resided. No difference in the early seasonal recrudescence of the hypothalamic GnRH, the testis volumes, the song production and the brain nuclei controlling song were found between the males of the populations (Caro et al. 2005a; Caro et al. 2005b; Caro et al. 2006). In females, however, the follicle growth periods were clearly distinct between the populations, and quantitative genetic analyses of laying dates demonstrated that the optimal breeding differentiation was driven by

the females (Caro et al. 2006). This demonstrated that assessing sex differences in cue perception, transduction and integration are essential components to take into account in phenological studies (Ball & Ketterson 2008) and that local adaptation to environmental heterogeneity could be a sexlimited phenomenon (Caro et al. 2009). Local population differentiations have also been demonstrated in other species, e.g. differences in (neuro-) hormones and gonadal cycles in male great tits (Silverin et al. 2008), rufous-collared sparrows (*Zonotrichia capensis*; Moore et al. 2005; Moore et al. 2006), in male and female stonechats (*Saxicola torquata*; Helm 2009) and between urban and forest blackbirds (*Turdus merula*; Partecke et al. 2004; Partecke et al. 2005).

Altogether, the understanding of phenotypic and genetic variation in the breeding phenology of the Mediterranean blue tits has required the close integration of most disciplines interested in phenological events, including evolutionary ecology, behavioural ecology, energetics, quantitative genetic, endocrinology and neurobiology. Although some genetic differentiation in the expression of neutral genetic markers, i.e. mini- and microsatellite loci (Dias et al. 1996; Charmantier 2000), and clock gene polymorphism have been described in these blue tit populations (Johnsen et al. 2007), the possibility of a seasonal clock differentiation mechanism between populations, and the genes involved, remain to be investigated (c.f. Liedvogel et al. 2009). Finally, if we want to predict adaptability to environmental variation it will also be critical to understand which environmental cues are important, how these cues are integrated in the HPG axis and how these proximate mechanisms are inherited between generations.

4. Outlook

Understanding the mechanisms underlying a specific phenology, which is constantly under natural selection, requires the integration of disciplines studying phenology from different angles, as outlined in §3 (Fig. 2.3). Here, we discuss a number of possible research questions that address less well-understood phenological topics in the context of avian reproduction for which we think it is pressing to make substantial progress in the coming years.

(a) Adapting to changing environments

One of the most obvious impacts of climate change on nature is the shift in phenology that has been observed in many taxa (Parmesan 2006), including the laying dates in birds (Crick & Sparks 1999). This phenomenon is what we would expect for any phenological trait that is phenotypically plastic and where temperature acts as a cue. However, the question is not whether these shifts occur but rather whether these shifts are adaptive: do organisms shift their phenology the exact amount they should? This seems rarely the case (Visser & Both 2005), as in many cases the shift in

phenology is either too weak or too strong to precisely match the shifts in phenology of other species in the food chain. This leads to mistiming, which may have severe fitness consequences for an individual (Visser et al. 1998; Thomas et al. 2001) as well as for population viability (Nussey et al. 2005; Both et al. 2006).

The paradox is thus that species are often phenotypically plastic in their phenology concerning temperature changes but that their response to increasing temperatures is not adaptive. This can be understood from Fig. 2.3. If climate change affects the environmental variables that serve as cues differently from the environmental variables that form the environment at the time of selection, then the response to climate change will no longer be adaptive. In other words, cues lose their predictive value. In extreme cases, the cues are not affected (birds relying on photoperiod in their overwintering area to depart to the breeding grounds) but the environment of selection is affected by climate change (the phenology of food for nestlings in the breeding area). A similar argument will also hold for resident species: climate change is unlikely to increase temperatures in a similar way throughout the year and such differential warming can cause an uncoupling of cues and the environment of selection (Visser et al. 1998): the cues perceived early on are no longer accurately predicting the future (Visser et al. 2004). Reaction norms may thus be no longer adaptive under climate change; animals have to be less or more temperature-sensitive than they are. This leads to natural selection on these reaction norms (Nussey et al. 2005), or in other words, to selection on the mechanisms underlying phenology.

For natural selection to operate on the response mechanisms underlying phenology, there needs to be genetic variation in these mechanisms (Nussey et al. 2005; Charmantier et al. 2008; Visser 2008). This makes the quest for genetic variation pressing. In many cases, physiological work is carried out on animals that are related to each other, but information on family relations is rarely used to estimate genetic resemblance, while no additional experiments would be necessary. Therefore, re-analysing existing data could be highly rewarding, provided that some pedigree data are available.

The absence of genetic variation in one or more components of the underlying mechanism could severely hamper micro-evolutionary change. In fact, at present there is very little evidence of such current micro-evolution in phenology (Gienapp et al. 2006), with perhaps a few examples such as the timing of diapause in the pitcher plant mosquito, which has shifted to longer photoperiods under a warming climate (Bradshaw & Holzapfel 2007). Ultimately, the rate of micro-evolution compared with the rate of climate change will determine the ecological impact of climate change (Visser 2008). Thus, a better understanding of the genetic variation in the response mechanism is essential.

(b) Integration of non-photic cues

In §3a, we have summarized the relatively well-understood effect of photoperiod on the mechanism underlying the phenology of avian reproduction. Effects of other environmental factors on the reproductive system have also been clearly demonstrated, in particular for social cues, food and temperature (reviewed by Lewis & Orcutt 1971; Hahn et al. 1997 ; Helm et al. 2006; Voigt et al. 2007; Silverin et al. 2008). However, little is known about how non-photoperiodic cues, as well as contributions from the circannual clock, are integrated to shape phenological events. Additionally, we know little about the dependence of cue integration on the state of an animal (e.g. its nutritional status) and whether reproductive status is a precondition to integrate certain cues.

The currently most comprehensive model of avian reproductive timing, developed by Sharp (2005), involves the circadian clock for daily time-measurement and two additional, interacting components. The first consists of the photoperiodically controlled GnRH-gonadotropin and vasoactive intestinal peptide (VIP)-prolactin neuroendocrine axes. These are complemented by the neuropeptide gonadotropin-inhibitory hormone (GnIH), which is regulated by the circadian hormone melatonin, and allows negative regulation of gonadotropin synthesis and release (Tsutsui et al. 2007; Perfito & Bentley 2009; Tsutsui 2009). An emerging body of research describes modulation of the GnRH system in response to photoperiodic, supplemental and social cues and thus supports its putative role as site of integration (e.g. Hahn et al. 1997; Moore et al. 2006; Ball & Ketterson 2008; Stevenson et al. 2008; Heideman & Pittman 2009; MacDougall-Shackleton et al. 2009). A second component of the model of avian reproductive timing comprises genotypedependent, neural inputs to, or intrinsic activities of, GnRH and VIP neurons (Sharp 2005), that could contribute to variation between individuals. So far our understanding of the mechanisms that drive phenology is mostly centred on photoperiodism and hampered by the scarcity of information on other cues. One of the problems with non-photic cues, in particular temperature, is that their actions are probably slower and more progressively integrated than cues such as photoperiod, which can induce rapid responses in the brain and elsewhere that can relatively easily be measured (using microarrays, immediate early gene expression or hormone concentrations; Meddle & Follett 1997). In addition, detection and transduction of non-photic cues is also much less well understood than that of photoperiodic cues.

Another challenge is to integrate ontogenetic effects on phenology into the physiological response mechanism. There is for instance a clear effect of photoperiod experienced by birds during rearing on their phenology of migration timing in the first year of life (Helm et al. 2005). Experiences during early adult lifetime (learning) can also affect the phenology of subsequent reproduction (Wingfield & Jacobs 1999), such as in blue tits where birds that bred too late in one year would advance their

laying date in the next year (Grieco et al. 2002). Lasting ontogenetic effects, for example those related to previous experience with environmental cues (Sockman et al. 2004), may have also confounded results of various experimental studies (Calisi & Bentley 2009).

(c) The role of the seasonal clock

The sensitivity of an animal to external cues involved in phenology depends on the seasonal clock. The detailed way in which the endogenous system responds to *Zeitgebers* appears to have evolved such that an optimal adjustment of seasonal activities to the environment is achieved. So far, however, evolutionary and ecological aspects of synchronization have barely been studied. This is partly owing to the difficulty of maintaining and observing sufficient numbers of captive organisms over at least a year. Recent studies on carpet beetles (*Anthrenus verbasci*; e.g. Nisimura & Numata 2001; Miyazaki et al. 2005) have revealed in detail how the circannual cycle of an animal interacts with photoperiod. Without external information, carpet beetles pupate in circannual intervals. The number of circannual cycles required for pupation is strongly affected by the quality of larval nutrition (Miyazaki et al. 2009). If provided with day length information the beetles synchronize pupation to the external year, but the timing response depends on when in their circannual cycle the Zeitgeber information is applied. Thus, the beetles either accelerate or delay pupation as described by a so-called 'phase response curve' (Miyazaki et al. 2005).

In our view, a phase response curve is a chronobiological, special case of a reaction norm. This idea, i.e. that annual activities are synchronized and adaptively modulated by photoperiod, has been pursued in studies of a passerine bird, the stonechat (e.g. Gwinner 2003; Helm 2009; Helm et al. 2009). This species is equipped with a circannual clock that persists for many cycles and is normally synchronized by the annual photoperiodic cycle. Comparative studies of populations from different locations revealed strong evidence for an innate basis of geographical differences in the responsiveness of certain phases of the circannual clock to its photoperiodic *Zeitgeber*. These differences can best be described in terms of population specificity in the reaction norm of the circannual oscillator to photoperiod. We suggest that further studies of adaptive modifications of circannual response mechanisms are an important step towards achieving a common evolutionary framework for the study of phenology (Fig. 2.3).

The mechanisms that underlie the seasonal clock and its interactions with environmental cues are only beginning to be explored (Lincoln et al. 2006). Early ideas that seasonal clocks arise as a summation of shorter processes, for example by 'counting' of circadian days (Gwinner 1986; Wikelski et al. 2008), or by a fixed sequence of interdependent physiological states (Mrosovsky 1970), has not been

supported. Instead, new studies point to long-term feedback processes on a tissue level (Lincoln et al. 2006) and to possible effects of energy turnover on the period length of the circannual clock, but these ideas still require further testing (Wikelski et al. 2008). The genetic basis of circannual rhythms is currently also unknown. A possible, underlying entraining mechanism could be in seasonal changes in the rhythmic expression of circadian candidate genes (e.g. Helfer et al. 2006; Martin et al. 2008; Davie et al. 2009). An example is the CRY–PER system (Cryptochrome–Period system; e.g. El Halawani et al. 2008; Hazlerigg & Loudon 2008). Expression of PER and CRY is based on a daily rhythm and locked to dawn and dusk, respectively. Therefore, changes in day length modify the interval between the expression peaks of PER and CRY and could thereby transduce information to the reproductive system (Paul et al. 2008). In spring, a shorter interval between CRY and PER may thereby contribute to entrainment of the circannual clock (Hazlerigg & Loudon 2008). However, these are speculative, early ideas about genes that may contribute to the functioning of the circannual clock (Notter & Cockett 2003; Notter 2008).

(d) Common experimental systems

Integration of approaches to phenology would be greatly accelerated if common experimental systems were developed or further expanded. All too often, ecologists experimentally study female egg-laying dates of a single wild population, physiologists study male gonadal size in the laboratory and researchers of phenology look at whole population shifts in timing on a large geographical scale. There are some examples of integrated study systems, like the white-crowned sparrow, which is studied in the wild and in the laboratory (Perfito et al. 2005), and the blue (see §3) and great tit (Drent et al. 2003). In the latter species even the same individuals are studied under controlled conditions in the laboratory as well as in the wild (Visser et al. 2009).

Setting up joined experiments is, in our view, mainly a matter of finding other researchers who are interested in a similar phenological trait but from a different perspective. Some species, like sparrows, juncos, starlings and great tits, are used by many different research groups and it would be highly feasible to do different measurements on the same individuals or at least on animals from the same population. This would be possible if researchers that need to obtain animals for their laboratory studies would be provided with nestlings from an evolutionary ecological population study and used these hand-reared animals for detailed studies on the underlying mechanism. This would bring together the physiological and ecological knowledge of animals from a single population. Moreover, as the birds used are related, with perhaps a known pedigree, this also opens up the possibility to look at genetic variation. We strongly advocate such approaches.

A historically grown discrepancy is the persistent difference between evolutionary ecologists and physiologists in the choice of the sex they work with (females versus males) and the environmental variable settings (4 versus 20°C difference between experimental groups, or a naturally increasing photoperiod versus transfer from 8 h to 16 h of light). But we are confident that there will be enough common ground, and enough to be gained, to overcome these problems.

(e) Concluding remarks

Phenology can be studied at the level of populations, but to understand how the year-to-year variation in the phenology of populations comes about, we will need to unravel more of the phenology of individuals and understand how the underlying mechanisms have been shaped by evolution. Individuals vary their phenology from year to year in response to cues from their environment, but are restricted by the possibilities of their evolutionary history, ranging from genetic variation, the make-up of circannual clock systems, to physiological pathways that form the basis of a certain phenotype. No matter from which discipline one approaches a problem related to phenology, it is important to keep in mind the complex relationships that underlie a phenological 'decision' like the laying of the first egg in spring. We have outlined an integrated framework to study phenology and we believe that this framework could function well to guide studies on a range of specific species and systems. It is not so much the lack of an existing framework as the subdivision of science that makes it hard to set up collaboration between scientists from different disciplines. Initiatives, such as the ESF/NSF/NSERC E-Bird network (Wingfield et al. 2008a), can play an important role in bringing researchers together, to get acquainted with each other's terminology and state-of-the-art. In our view, the benefits of close collaboration, in particular in combined studies (see §4d), will outweigh the costs of setting up such studies.

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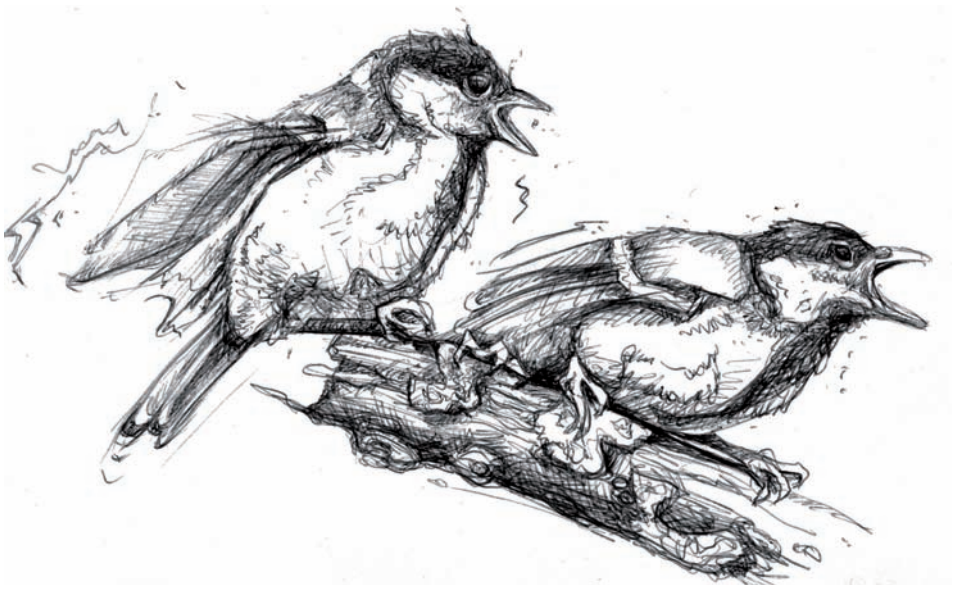
Chapter 3

Marcel E. Visser, Sonja V. Schaper, Leonard J.M. Holleman, Alistair Dawson, Peter J. Sharp, Phillip Gienapp and Samuel P. Caro

Genetic variation in cue sensitivity involved in avian timing of reproduction



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Abstract

1. Annual variation in the timing of avian reproduction is associated with predictive cues related to ambient temperature. Understanding how these cues affect timing and estimating the genetic variation in sensitivity to these cues, is essential to predict the micro-evolutionary changes in timing which are needed to adapt to climate change.

2. We carried out a 2-year experiment with great tits (*Parus major*) of known genetic background, which were kept in pairs in climate-controlled aviaries with simulated natural photoperiod and exposed to a seasonal change in temperature, where the two treatments differed by 4°C. We recorded the dates of laying the first and last eggs and timing of moult, as well as physiological proxies associated with reproduction: plasma luteinizing hormone (LH), prolactin and gonadal size at four-weekly intervals.

3. The temperature treatments did not affect first-egg dates, nor gonadal growth or plasma LH and prolactin concentrations. However, birds terminated egg laying, regressed their testes and started their moult earlier at higher temperatures.

4. There were marked family differences in both the start of egg laying, with sisters from early laying maternal families laying early, and in the termination of laying, indicating that there is heritable variation in sensitivity to cues involved in timing.

5. Our experiment, the first to use genetically related individuals in an experimental design with a natural change in photoperiod and biologically realistic temperature differences, thus shows that genetic adaptation in cue sensitivity is possible, essential for species to be able to adapt to a warming world.

Introduction

In many organisms, seasonal timing of reproduction or growth has major fitness consequences (Perrins 1970; Nussey et al. 2005; van Asch & Visser 2007). In a seasonal environment, varying environmental conditions determine an optimal period for most life cycle events including reproduction. Therefore, initiating reproduction as little as a few days early or late can already reduce fitness in terms of increased parental effort, reduced offspring quality or offspring survival (Visser et al. 1998; Thomas et al. 2001). The optimal time for the onset of reproduction commonly varies from year to year as the optimal conditions for breeding are often set by the phenology of other species in the food chain. Such variability in environmental conditions selects for phenotypic plasticity (Gomulkiewicz & Kirkpatrick 1992; Kawecki & Stearns 1993). Many species are phenotypically plastic (Pigliucci 2001) in their seasonal timing and consequently the same individual can start breeding at different times in different years. To make an informed decision on the timing of reproduction, individuals use a number of abiotic and biotic cues to predict future conditions (Wingfield & Kenagy 1991), but with the exception of photoperiod, very little is known about which cues provide reliable information. While photoperiod has been shown to strongly affect

seasonal timing in many species, including mammals and birds (Rowan 1926; Follett et al. 1985; Dawson et al. 2001; Goldman 2001; Sharp 2005) this cue cannot account for the year to year variation in phenological timing as there is no year-specific information conveyed. In temperate zones, the optimal conditions for reproduction for a wide range of organisms, including plants, invertebrates and vertebrates alike, are often directly or indirectly affected by temperature, and thus it is likely that animals rely on cues that are strongly correlated with temperature and/or temperature itself.

The predictive cues animals use to time breeding can ultimately only be studied under controlled environmental conditions, as for example temperature is correlated with changes in both climate and seasonal development of vegetation. Besides correlational evidence from the field (Nager & van Noordwijk 1995; McCleery & Perrins 1998), there have been a number of experiments that have investigated the direct effect of temperature as a cue, concentrating on avian egg laying as a measure of reproduction. Meijer et al. (1999) worked with three groups of captive starlings (*Sturnus vulgaris*) and increased ambient temperature by 5°C at different periods in spring. In each of the groups, on average the first egg was laid 7-8 days after the increase in temperature. Salvante, Walzem & Williams (2007) studied egg laying in captive zebra finches (*Taeniopygia guttata*) either kept at 7°C or 21°C and showed that the birds under cold conditions significantly delayed their egg laying. More recently, Visser, Holleman & Caro (2009) showed that great tits (*Parus major*) responded to temperature in their timing of laying. Using climate-controlled aviaries that housed independent pairs of great tits, they mimicked the spring temperature progression of a cold and a warm year, thereby using realistic, and thus small, differences in temperatures between treatments. Despite the small difference in temperature, birds from the cold treatments delayed the onset of egg laying (Visser et al. 2009).

Global climate change is currently affecting temperatures world-wide and in many species there is a shift in spring phenologies (Parmesan 2006). However, often the shift in timing in one species within a food chain differs from the shift in timing of another species (Visser & Both 2005; Thackeray et al. 2010) and thus the inter-related seasonal timing of many species is no longer matched with that of their prey food which can lead to severe fitness consequences (Visser & Holleman 2001; Visser et al. 2004; Both et al. 2006). Given that the suite of physiological and behavioural mechanisms underlying reproduction is often heritable (Ronning et al. 2007; Williams et al. 2009), albeit sometimes low (Mousseau & Roff 1987; Merilä & Sheldon 2000), and that there is strong selection on the timing of reproduction, micro-evolution of timing may take place and potentially restore synchrony within a food chain (Visser 2008).

To predict micro-evolution in seasonal timing, for example in the timing of the onset of laying, it is essential that we establish whether there is genetic variation in laying date (Sheldon et al. 2003; Nussey et al. 2005; Caro et al. 2009). Laying date is the outcome of a cascade of endocrine and behavioural processes, which in birds starts

months previously with the onset of gonadal growth (Ball & Balthazart 2002), and thus variation in laying date can be caused by individual variation in a wide range of underlying processes (Visser et al. 2010a). However, no systematic attempt has been made to determine in which way individuals within a population differ in their physiological mechanisms underlying timing of reproduction (Wingfield et al. 2008), therefore restricting our understanding of the evolutionary potential of organisms (Chown et al. 2010). If birds differ genetically in their physiological or behavioural sensitivity to predictive cues this would mean that natural selection will favour individuals that show a higher sensitivity to predictive cues, which will lead to micro-evolution in cue sensitivity. Alternatively, variation among individuals in laying date can be caused by genetic differences in the energetic costs associated with laying date (Perrins 1970; Stevenson & Bryant 2000; Chastel et al. 2003) and in that case natural selection will act on the genetic variation in these energetic costs and not in cue sensitivity. By comparing laying dates between birds exposed to different cues, but with *ad libitum* food, we can determine if birds differ genetically in cue sensitivity or the way these cues are transduced via physiological pathways.

In this paper we study great tits in climate-controlled aviaries under two temperature regimes and a simulated naturally increasing photoperiod. We used pedigreed offspring from a wild population with genetic differences in reproductive timing by selecting broods from early and late laying maternal lines or 'families'. In addition to determining the onset of reproduction, we also recorded its termination (last egg date and regression of gonads) and the onset of post-nuptial moult because these are well-established to be affected by temperatures (Dawson 2005a; see Appendix 3.4). Furthermore, we determined which components of the physiological cascade leading to the onset of laying were affected by environmental cues by measuring reproductive hormones [luteinizing hormone (LH) and prolactin; see Appendix 3.3] and gonadal development in males and females at regular intervals.

We thus integrated evolutionary ecology and reproductive physiology to determine whether there is heritable variation in responses to cues for the timing of seasonal breeding. This is the first time that this topical question has been addressed. While we have shown that great tits' laying date is affected by temperature (Visser et al. 2009) we did not study the genetic background of this effect. And where we showed a genetic effect in plasticity of timing of reproduction, we used data from wild great tits (Nussey et al. 2005) and were thus not able to distinguish between genetic variation in cue use and/or energetic constraints. Furthermore, all other studies on effects of temperature in females on timing use acute and large changes in photoperiod and large differences in temperature among treatments, thus deviating from a setup that resembles a natural situation.

Our aims were thus to determine 1) whether there is genetic variation in both onset and termination of reproduction (which would indicate that variation among

individuals is genetic variation in cue sensitivity, given that animals have *ad libitum* food), 2) the effect of temperature on the onset of reproduction (laying dates), 3) the effect of temperature on the termination of reproduction and the onset of moult, 4) the genetic variation and the effect of temperature on reproductive physiology (gonad size, hormone levels) to get a better understanding where in the physiological cascade temperature may play a role.

Materials and Methods

Aviaries

Great tits were kept in pairs in 36 separate climate-controlled indoor aviaries (2 x 2 x 2.25 m) under an artificial light regime mimicking a natural daylight pattern (see also Visser et al. 2009). Photoperiod was increased twice a week following the natural increase in day length (i.e. from 7.45L:16.15D to 16.30L:7.30D at the winter and summer solstice, respectively at 52°N). The main source of light was three high frequency fluorescent light tubes, but half an hour before these were switched on, and half an hour after they were turned off, a 8 W light bulb mimicked dusk and dawn. The birds were fed *ad libitum* with a constant daily amount of food consisting of a mixture of minced beef, proteins and vitamins, sunflower seeds, fat, a mix of dried insects, a mixture of proteins, vitamins, minerals and trace elements, (CéDé-mix), a surplus of calcium and water for drinking and bathing. Two nest boxes were provided per aviary, nesting material consisting of moss and dog hair was provided from March onwards.

Birds

Two sets of 36 male-female pairs of great tits were used for an experiment that was replicated in 2006 and 2007. Nestlings were taken as whole broods (with known laying dates) at day 10 after hatching from our long term study population (1955-present) at the Hoge Veluwe (the Netherlands) in 2005 (collected between 38th and 56th April) and 2006 (collected between 47th and 59th April, the average laying date of the entire Hoge Veluwe population was 8.5 days later in 2006 than in 2005). In the field, females incubating eggs were identified by their unique colour ring combination which was put on either the previous breeding season or in the winter roosting inspections. The broods that we selected from the entire population were both from early and late reproducing families to include genetically different birds in the experiment. Each year, we selected five broods with an early laying date and for which these females (and as much as possible also the females' mothers) were also among the earliest broods in previous years. Similarly, we selected five late first broods (i.e. avoiding replacement and second broods) each year. A blood sample (2-5 µl) was taken from all nestlings at day 2 after hatching, as well as from their parents at day 7 after hatching. Using these samples we determined the sex ratio of the nestlings (Griffiths et al. 1998)

and whether or not the nest contained extra-pair chicks (Saladin et al. 2003) before taking the nestlings in at day 10.

The nestlings were taken to the Netherlands Institute of Ecology (Heteren, the Netherlands), where they were weighed (day 10 weights) and hand-raised until independence (Drent et al. 2003). After fledging, birds were kept in single-sex groups in large (2 x 4 x 2.5 m) outdoor aviaries until 1st December 2005 or 2006, after which they were housed as opposite-sex pairs in indoor climate-controlled aviaries (2 x 2 x 2.25 m) for experiments to be conducted the following year. We mated 'early males' with 'early females' and 'late males' with 'late females', avoiding brother-sister pairings. In 2007 we were short of males so three males of the 2006 experiment were used again, but given this small number of 2-year old birds, we did not include male age in our analyses. The experiment ended at the end of August after which the birds were used for additional experiments (Caro & Visser 2009; Helm & Visser 2010).

Treatments

The 36 pairs of great tits were divided into two groups that differed in the ambient temperature treatment to which they were exposed. The temperature values used were close to typical temperatures for the season in the Netherlands (see Fig. S3.1), with the high temperature set to be always 4°C higher than the cold temperature treatment (but see below for the actual realized temperature differences). From 1st December to the end of February temperatures were kept constant at 4 and 8°C respectively, after which temperatures gradually increased up to 1st July, reaching 15 and 19°C respectively (an average increase of 0.65°C/week). The temperature difference of 4°C was maintained during the moulting period (see Fig. S3.1). Temperatures were controlled ±0.5°C by either heating or cooling the air that was circulated in the aviary. The realized temperatures were recorded in each of the aviaries every 10 min.

A difference of 4°C was chosen as this is the difference between a very cold and a very warm year in the Netherlands for the period of the year for which the temperatures best correlate with laying dates in the wild (16th March-20th April; Visser et al. 2006). We used a gradual increase, with a fixed difference between the two treatments rather than an actual pattern of temperature fluctuations (as was used by Visser et al. 2009). The main reason for this is that actual temperature patterns display temporal autocorrelation, and this makes it difficult to assess in which period temperature matters. Using a fixed temperature difference, this difference between treatments is the same for any period (but see above) and hence for the statistical analysis there is no need to select a specific period over which the average temperature is used.

The realized temperatures differed from the target temperatures as especially the low target temperatures in December and January (cold treatment 4°C, warm treatment 8°C) could not be realized. From February onwards the differences in temperature

treatment were clear (between 2.5 and 3.5°C) although still lower than the 4°C that we aimed for. In all analyses we either used treatment (warm vs. cold) or the realized temperatures as explanatory variables.

Measurements

In the breeding season nest boxes were checked daily and the date that the first egg was found recorded as the laying date. Eggs were weighed (to the nearest 0.01 g) and measured (to the nearest 0.01 mm) on the day they were laid, stored at -80°C and replaced by dummy eggs. After the birds laid the last egg the clutch size was determined. After 5 days of incubation the clutch and nest were removed to allow the birds to rebuild and start a new clutch. This procedure was repeated until no more clutches were initiated. The date of the last egg of the last clutch was the last egg date (termination of reproduction). Loosing clutches and re-nesting is common for great tits in nature.

In 2006, once every 4 weeks a blood sample (75 µl) was taken from the wing vein for luteinizing hormone (LH) measurements (9 out of 36 aviaries per week). In 2007, once every 4 weeks the birds (all aviaries on a single day) were blood sampled (75 µl) from the wing vein for prolactin measurement (see Appendix 3.3 for the methodology of hormone assays). Also in 2007, in an alternating pattern with the blood sampling (i.e. also once every 4 weeks), all birds were laparotomized to measure gonadal development, males during the entire breeding season, females only up to egg laying in order not to interfere with the egg laying process.

Birds were unilaterally laparotomized under anaesthesia with isoflurane (Forene; Abbott, Hoofddorp, the Netherlands). Left testis length and width and diameter of the largest developing follicle in the ovary were measured to the nearest 0.1 mm, using a scale engraved in the ocular of a binocular microscope. Testis volume was calculated using the equation: $V=4/3\pi a^2 b$ where a is half the testis width and b is half the testis length. Follicle volume was calculated using the equation: $V=4/3\pi a^3$ where a is half the follicle width. In both years, moult of the primary wing feathers was scored once every 4 weeks from the end of breeding onwards (see Appendix S3.4). Preceding the experiment in the climate-controlled aviaries all birds were tested for their exploratory behaviour at 6 weeks after independence using a novel environment test developed by Verbeek et al. (1994) and from which an exploratory score was calculated following procedures outlined by Dingemanse et al. (2002). Exploratory behaviour is used as a proxy for personality in great tits (Drent et al. 2003).

Statistical analyses

Our primary interests were the effects of the genetic background and temperature on the onset and termination of reproduction, which were analysed using mixed

effects Cox proportional hazards models (see Appendix 3.1). The models allowed us to use an iterative procedure, based on a linear predictor, for the calculation of a temperature variable that incorporates the current, as well as earlier temperatures experienced (for details, see Gienapp et al. 2005). A weighing factor α assesses the relative importance of current versus earlier temperatures: if this factor is large, the model places a high weight on the most recent temperature (the temperature variable resembles the mean temperature of that particular day), while if it is small, previous temperatures are given more importance, which implies a longer 'memory' of previous temperature conditions experienced.

We analysed the data in three steps: 1) we used treatment (2 levels: warm/cold) and family (20 levels) as explanatory variables (fixed factors), 2) we used actual temperatures (including within-treatment variation in temperature over time) rather than treatment, together with family, as explanatory variables, and 3) we replaced family with mother's laying date (as a measure for early/late families) and included additional characteristics of individual birds (personality and weight at day 10). In this last step, family was included as a random effect and year was included as a two level fixed effect. The models allowed us to include an interaction term between temperature and day length. Because day length is almost linearly related to date and did not vary between years, it is not possible to include it as main effect and it was only fitted as an interaction term.

The gonadal size (log volume) was analysed in a mixed model with sample period (date), treatment (warm/cold) and sex as fixed effects and bird identity as a random effect. Clutch size of first clutches was first analysed in a GLM with year, treatment and family as fixed effects to look for family effects on clutch size. Next, we analysed the effect of temperature treatment on the relationship between clutch size and laying date in a mixed model with laying date, year and treatment as fixed effects and breeding pair as random effect (to account for multiple clutches per pair).

For all mixed models we used the c.1. PROC GLM MIXED in SAS 9.1, using Sattherwaite's method of calculating degrees of freedom (df), for all GLM we used PROC GLM in SAS 9.1. In all models we used normal distributed errors and a step down approach where we removed non-significant terms ($P>0.05$), starting with the interactions until a model remains where all main effects and/or interactions are significant.

Results

Onset of egg laying

In 2006, 34 out of 36 pairs laid eggs, and in 2007, 28 out of 36 pairs laid. The probability of laying was not dependent on treatment, family or individual characteristics such as weight at day 10 (GLM with binomial errors). There was a striking difference in

mean laying date between the 2 years, despite the fact that the same experimental protocols were used. (2006: 28.9 April, 2007: 10.7 May). Size of first clutches (42 pairs) was not correlated with treatment nor family, but differed significantly between years [$F_{1,40}$=4.28, P=0.045; mean clutch size for 2006: 12.3 (SE=1.02) and for 2007: 9.48 (SE=0.93)]. In an analysis on 101 clutches produced, there was a significant effect of laying date ($F_{1,73}$=65.40, P<0.0001) but not of year ($F_{1,44.4}$=1.95, P=0.17) nor temperature treatment ($F_{1,44.5}$=0.85, P=0.37): clutch size decreased by 0.12 egg for every day the clutch initiation was delayed.

The statistical model could not handle a single model incorporating the effects of both the females' family and the males' family and consequently this was done using separate models. The onset of laying was not affected by temperature treatment, in either male or female family analyses (in model with female family and treatment: χ^2=0.12, 1 df, P=0.72; in model with male family and treatment: χ^2=1.28, 1 df, P=0.26).

Temperature, after finding the best temperature value α (see Appendix 3.2) was not significant in models including female or male family (Table 3.1a). However, laying date was significantly different between families, in the analysis of both female and male families (Table 3.1a). Thus, sisters as well as brothers' mates resemble each other in their timing of reproduction more than unrelated individuals, which indicate that there is heritable variation in the timing of egg laying.

Note that as there were only three males which were used in both years (and none of the females) all year variance is attributed to family. This, however, does not account for the amongst family variation, since in the within-year analyses family is (sometimes nearly) significant (Table 3.1a).

When we replaced family by quantified characteristics of individual males and females in the analysis and added female family as a random effect, temperature was still not significant, neither as a main effect nor as an interaction with photoperiod or laying date of the female's mother (Table 3.1b). Individuals varied in their characteristics [range for 10 day old nestling weights females: 8.6-17.4, mean of 14.4, for males: 7.8-18.6, mean of 15.0; variation in personality over the entire range (2.7-42.3)]. However, despite this variation, none of the individual characteristics (nestling weights or adult personalities) of the males or females had a significant effect on laying date (Table 3.1b).

However, there were (in addition to year) significant family characteristics: both the laying date of the female's mother and the laying date of the male's mother influenced laying dates in the aviaries (Table 3.1b; Fig. 3.1a-d). While daughters from early mothers were more likely to lay early, sons from early mothers had mates that laid later than expected on the basis of their own mothers' laying date (see also Discussion).

Table 3.1: The onset of reproduction (laying date) of pairs of great tits kept at two temperature treatments in an experiment replicated over two years. Data were analysed using mixed effects Cox proportional hazards models (see Appendix 3.2) using (a) family and temperature as explanatory variables, or (b) using year, temperature and individual characteristics of the male and female as explanatory variables. Analysis (a) was run separately for male and female family. Also, in (a) the family effect was tested for the 2 years separate (see text). For the temperature variable the weighing factor α is reported: if this factor is large, the model places a high weight on the most recent temperature, if it is small, it implies a longer 'memory' of previous temperature conditions (see Appendix 3.2).

a. Temperature and family

Explanatory variable	χ^2	df	P
Females			
Temperature (α=0.01)	0.57	1	0.45
Family	44.0	19	**<0.001**
Family 2006	14.97	9	0.09
Family 2007	19.84	9	**0.019**
Males			
Temperature (α=0.01)	0.27	1	0.60
Family	33.54	18	**0.014**
Family 2006	17.65	10	0.061
Family 2007	22.51	9	**0.007**

b. Temperature and individual characteristics

Explanatory variable	χ^2	df	P
Year	4.47	1	**0.034**
Temperature (α=0.10)	0.37	1	0.54
Temperature*photoperiod	1.29	1	0.26
Temperature*LD female's mother	0.03	1	0.87
Laying date of female's mother	5.42	1	**0.019**
LD female's mother*year	3.00	1	0.083
Laying date of male's mother	4.77	1	**0.029**
Female personality	2.26	1	0.13
Female weight at day 10	1.58	1	0.21
Male personality	0.06	1	0.81
Male weight at day 10	1.68	1	0.19

There was no effect of temperature on the development of the gonads in either males or females (Fig. 3.2). There was, however, in addition to the obvious date effect, an effect of male family (mixed model with bird identity as random effect: $F_{11,131}$=2.22, P=0.017): brothers are similar to each other in their testis sizes. No such effect was found for female family ($F_{9,133}$=0.84, P=0.58). We found no clear effect of temperature or family on LH or prolactin levels (see Appendix 3.3).

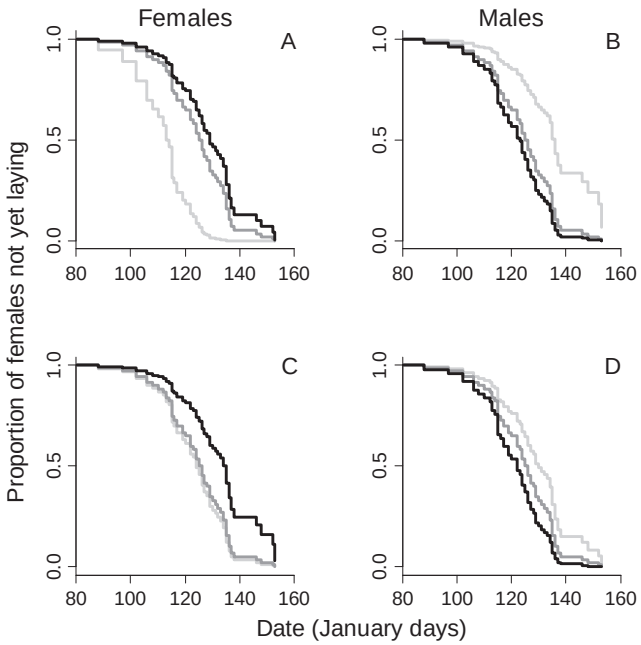

Figure 3.1: Curves for the proportion of pairs of great tits not yet laying for the January date (number of days since 31st December) indicated, i.e. curves that descent early represent birds that lay early. Curves differ for female (A and C) and male (B and D) mothers' laying date (see Table 3.1 b). Dark grey lines indicate birds from early laying families (25 percentile of laying date of the mother), black lines indicate the median laying date (which happened to be identical for both years) and the light grey lines indicate birds from late laying families (the 75 percentile of the mothers' laying date). The curves illustrate the outcome of the model in Table 3.1 b in which, in case of (A) and (C) the laying date of the male's mother, and in case of (B) and (D) the female's mother's laying date was set to the median. Panels (A) and (B) represent 2006, panels (C) and (D) represent 2007.

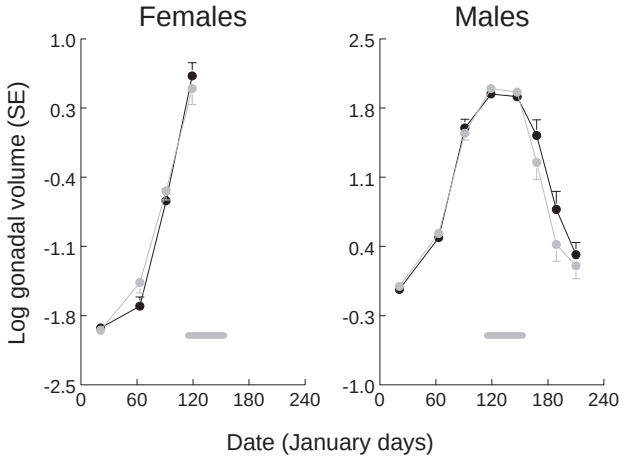

Figure 3.2: Log gonadal volume (SE) of male and female great tits kept at two temperature treatments in an aviary experiment in 2007. Grey points represent the warm treatment, the black points the cold treatment. The grey horizontal bar indicates the range of clutch initiation. Date in January days (i.e. the number of days since 31st December).

Termination of reproduction

As for the onset of reproduction, the statistical model of the termination of reproduction could not handle a single model incorporating both the females' family and the males' family. Consequently the analysis was done using separate models. The termination of reproduction was clearly affected by treatment, both in male and female family analyses (in model with female family and treatment: χ^2=14.97, 1 df, P=0.0001; in model with male family and treatment: χ^2=8.64, 1 df, P=0.003). There was an effect of temperature on the termination of laying (Table 3.2a): birds in warmer aviaries terminated reproduction earlier than birds in colder aviaries. In addition, sisters from different families as well as brothers from different families varied in their termination of reproduction (Table 3.2a) and these family effects largely persisted when analysed for each year separately (to exclude the confounding between-year variation; Table 3.2a). Neither year nor any of the family or individual parameters for females or males significantly affected the last egg date (Table 3.2b). Only temperature as a main effect remained in the model (Table 3.2b). Overall, at higher temperatures reproduction was terminated earlier (see Fig. 3.3).

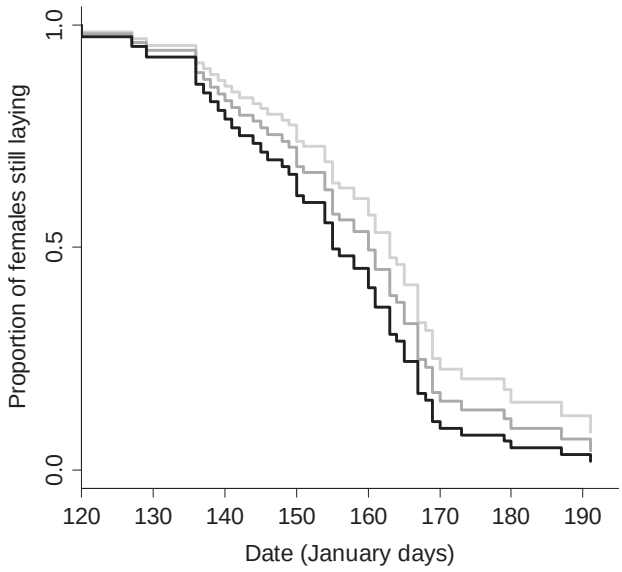

Figure 3.3: Curves for the proportion of pairs of great tits not yet stopped laying for the January date (i.e. the number of days since 31st December) indicated, i.e. curves that descent early represent birds that terminate reproduction early. Curves differ for the temperature the birds experienced. Temperatures were calculated as mean daily temperature weighted with a weighing factor α (see Appendix 3.2) of 0.01; the dark grey line indicates the 25 percentile lowest temperatures, the black line the median and the light grey line the 25 percentile warmest temperatures. The curves illustrate the outcome of the model in Table 3.2 b.

Table 3.2: The termination of reproduction (last egg date) of pairs of great tits kept at two temperature treatments in an experiment replicated over 2 years. Data were analysed using mixed effects Cox proportional hazards models (see Appendix 3.2) using (a) family and temperature as explanatory variables, or (b) using year, temperature and individual characteristics of the male and female as explanatory variables. Analysis (a) was run separately for male and female family. Also, in (a) the family effect was tested for the 2 years of the experiment separately (see text). For the temperature variable the weighing factor α is reported (see Appendix 3.2).

a. Temperature and family

Explanatory variable	χ^2	df	P
Females			
Temperature (α=0.10)	25.42	1	**<0.0001**
Family	46.79	19	**<0.0005**
Family 2006	25.09	10	**0.003**
Family 2007	17.49	9	**0.042**
Males			
Temperature (α=0.01)	23.61	1	**<0.0001**
Family	34.85	18	**0.01**
Family 2006	24.05	10	**0.007**
Family 2007	10.52	9	0.31

b. Temperature and individual characteristics

Explanatory variable	χ^2	df	P
Year	0.38	1	0.54
Temperature (α=0.01)	6.75	1	**0.01**
Temperature*photoperiod	0.01	1	0.94
Temperature*LD female's mother	0.31	1	0.57
Laying date of female's mother	3.43	1	0.06
LD female's mother*year	0.01	1	0.97
Laying date of male's mother	1.44	1	0.23
Female personality	0.19	1	0.66
Female weight at day 10	0.01	1	0.97
Male personality	1.39	1	0.24
Male weight at day 10	0.01	1	0.92

Gonadal regression was only assessed for males (Fig. 3.2). In week 21 and 24 there was no effect of temperature treatment on gonadal size but in week 27 the testes of males in warm aviaries were smaller than those of males in cold aviaries ($F_{1,23}$=4.60, P=0.04; estimates cold: 0.64 mm^3 (SE=0.21) and warm: 0.31 mm^3 (SE=0.20). Similar to the termination of reproduction, the onset of moult was clearly affected by temperature treatment and the duration of moult was in turn strongly affected by the onset of moult (see Appendix 3.4): for every day a bird started moulting later, the duration of moult decreased with half a day. On top of this effect, females, which also started later, moulted on average 6 days faster.

Discussion

In this integrative study we investigated how variation in genetic background and in temperature influence the breeding phenology of great tits, which in the Netherlands currently faces the detrimental effects of a warming world on breeding success (Nussey et al. 2005). We found no effect of a moderate temperature difference of 4°C on pre-breeding physiology (gonadal development, LH, prolactin) or on the onset of egg laying, but we did demonstrate that increased ambient temperature advanced the date of the last egg laid, the onset of male gonadal regression and the onset of moult. We also found clear differences between families, both in terms of the onset and the termination of reproduction, indicating that there is genetic variation in cue sensitivity. Females that have mothers that lay early in the wild laid early themselves in the aviaries, but in males this was the opposite: sons of early laying mothers, which were always paired up to early females, had female mates which laid late (see below). Similarly, some families had larger testis volumes than others and this difference was influenced by the temperature treatment to which they were exposed. This constitutes, to our knowledge, the first demonstration that testis size variation is partly explained by genetic (heritable) components in birds. Finally, we found a large unexplained deviation in mean laying date between the 2 years of the experiment (see below).

Genetic effects

There were clear family effects on the timing of reproduction under *ad libitum* food conditions which indicates that there is genetic variation in cue sensitivity (which may be temperature or photoperiod in our experiment). This genetic variation is essential for our understanding of how natural selection may lead to an evolutionary change in timing of reproduction. If the genetic variation in laying date, as found in the wild, originated from variation in the energetic costs associated with laying eggs, we would not have observed that in our experiment where birds are fed *ad libitum*. In that case, where genetic variation in egg production capability determines variation in laying date, selection on cue sensitivity would not lead to an evolutionary response and hence would lead to negative demographic consequences due to the sustained selection (Visser et al. 1998; Visser 2008).

We interpret the family effects on the onset and termination of reproduction as genetic effects but we cannot rule out effects of a shared early environment: all eggs within a clutch were laid and incubated by the same female and were reared by their parents in the wild up to day 10. After that, they were hand-reared under standardized conditions. While weight on day 15 is an important indicator of chick survival in the wild (Verboven & Visser 1998), we found no effects of the nestling weight on day 10 on reproductive timing as an adult (Table 3.1b). So even if we cannot rule out other common environment effects, we strongly believe in a genetic component causing differences in timing.

The family effect in females was due to the effect of the mother's laying date: females in the experiment laid earlier if their mother laid earlier in the wild (Fig. 3.1a,c). In males this effect was opposite: males with an early laying mother had mates that laid late in the aviaries given the laying date of their own mother (and after correcting for the effects of the female's mother's laying date; Fig. 3.1b,d). This is a puzzling result. However, a very similar effect was found in Visser et al. (2009). There, laying dates from the same individuals (rather than from parents and their offspring as in the current experiment) were obtained from the wild and from the aviaries, but in females and males, laying dates in aviaries were respectively positively and negatively correlated with laying dates in the field (Visser et al. 2009). A possible explanation could be that males that have early laying mates in the wild are more active and aggressive. While in the wild this may result in earlier or more active courtship, in captivity this aggression may inhibit the onset of egg laying in their mates. However, we did not find an effect of male personality, which we know is correlated to aggression (Verbeek et al. 1996), in our study.

In the analysis of the hormones LH and prolactin (see Appendix 3.3), which are associated with reproduction, we did not find any family effects which may indicate that there is no genetic variation in the endocrine pathway underlying timing of reproduction. If this finding is confirmed it would mean that natural selection cannot act on this part of the endocrine pathway.

Temperature effects

Contrary to the results of earlier experiments in the same aviaries (Visser et al. 2009), we found no effect of temperature treatment on laying date in the present study. The temperature treatments were however very different between the two sets of experiments. In Visser et al. (2009) the temperatures mimicked those of two specific years, characterized as either being especially cold or warm, which were chosen based on the laying date of wild great tits in these years. Laying dates in Visser et al. (2009) correlated with the mean ambient temperatures in the period 21st April-10th May. In the current experiment, we used a constant difference of 4°C between the two temperature treatments (see Fig. S3.1) which offers the advantage that over any period the mean temperatures differ by a fixed quantity. When we compare the realized temperature for the period from 21st April-10th May, there was a clear difference: 2006 warm: 15.2°C, cold: 12.6°C; 2007 warm: 15.1°C, cold: 12.5°C. This is an overall difference of 2.5°C, which may seem small (and in fact was smaller than we aimed for) but it was large enough to affect the later stages of egg laying. Moreover, from what we know from our wild population, such a temperature difference in certain periods of spring is ecologically relevant and based on the estimates for the temperature effect in Visser et al. (2009), should have led to a difference in laying date of about 30 days. Clearly, the temperature effect of the treatments in Visser et al. (2009) was not captured in the temperature treatments in this experiment.

The reason why we found no effect of the temperature treatment remains unclear. When compared to the temperature patterns of Visser et al. (2009) a potential explanation is the lack of temperature variation, both on a daily scale and a seasonal scale. Another explanation could be the identical rate of temperature increase in the two treatments. If it is temperature increase rather than absolute temperature that is a cue for timing of reproduction then we would not expect to find any temperature effect in the experimental setup we used. From experiments manipulating photoperiods in starlings, we know that passerine birds can be more sensitive to variation in environmental cues than in their absolute levels (Dawson 2005a). Obviously, these two explanations need to be tested in future experiments. The lack in our understanding in how environmental variables affect laying date is also illustrated by the large differences in mean laying dates between 2006 and 2007. The temperature differences between these 2 years in the period 21st April-10th May were very small, again not confirming the effects of temperature on laying date as described in Visser et al. (2009). When comparing the temperature patterns of these two years (see Fig. S3.1) there was a stronger increase in temperature in 2006 in both treatments between January days 50-90 (1=1st January). This would indeed indicate that the hypothesis that it is the increase in temperature rather than the absolute temperatures that plays a role in timing is worth pursuing.

We see three other potential explanations for the difference in mean laying dates between 2006 and 2007. First, a potential explanation is that the between-year variation in the aviaries might have been affected by outside conditions such as for instance air pressure patterns. In the wild, 2006 was a relatively late year (mean laying date of great tits at the Hoge Veluwe 24.7 April) while 2007 was an early year (14.4 April). Secondly, we carried out laparotomies at four-weekly intervals in 2007 while in 2006 we only did a single laparotomy per bird in January (from which the measurements were too inaccurate to be used). While we cannot exclude this explanation we do want to point out that it is the early mean laying date in 2006 which is an outlier. In the experiments of 1999-2005 the mean laying dates were as late as in 2007. Thirdly, the birds may differ from year to year, related to the conditions in the wild prior to moving to captivity. As the great tits on the Hoge Veluwe laid on average 8.5 days earlier in 2005 compared to 2006, the birds used in the 2006 experiment were taken in 9 days earlier for the earliest broods and 3 days earlier for the later broods. This will affect for instance the photoperiod they experienced in the field (a 9 day difference in mid-May is a difference of 25 min of day length). It is unlikely that this difference will significantly affect the laying date of the birds in their first breeding season but we cannot rule this out.

Interestingly, the earliest birds laying in 2006 were in the cold treatment, which could be an indication that these birds were anticipating two broods and therefore laid their first clutch early (c.f. Visser et al. 2003). As there is no correlation between the laying

date and the date of the last egg (2006 cold treatment data set: n=18, Pearson r = -0.15, P = 0.56) these early laying birds indeed keep on laying for a longer time but not longer than birds that start laying later, thus we find no support for this hypothesis.

Temperature had a clear effect on the termination of reproduction. Birds in the warm treatment stopped laying earlier, males regressed their gonads earlier and birds started moulting earlier (see Appendix 3.4 for a full discussion). Although the causation of the effect of temperature on the termination of breeding remains to be discovered, it must be stressed that the activity of the reproductive axis is very sensitive to temperature in the late phases of breeding. Other studies have shown a similar effect of temperature on testicular regression associated with the development of photorefractoriness (Dawson 2005a) but none have included observations on the termination of egg laying. The temperature treatments often differed by 10-15°C, while in the present study we show the same effect with a realized temperature difference of only 2.5°C. Our results are consistent with data from the wild great tit population where the aviary birds originating from (Hoge Veluwe) where fewer pairs produce a second brood in warmer years (Husby et al. 2009). Reproduction is terminated earlier, probably because their caterpillar food becomes unavailable sooner and temperature can be used as a cue to signal this.

Concluding remarks

It is not clear why temperature effects on the sexual recrudescence and onset of laying are so difficult to demonstrate experimentally and produce so many inconsistent results, while there is ample evidence from field data that temperature does affect laying dates (e.g. Dhondt & Eyckerman 1979; Perrins & McCleery 1989; Nager & van Noordwijk 1995; Charmantier et al. 2008). First, there is a lack of data on laying dates in captivity, especially following temperature manipulation. Secondly, there is clear evidence that temperature effects on birds' breeding phenology are very subtle and interact with other cues. For example laying is influenced by a change in temperature in the few days that directly preceded egg formation (Kluyver 1951; Meijer et al. 1999). In contrast, most experiments manipulating temperature in captivity also involve acute transfers from short to very long photoperiods or large differences in temperature, and both cues were generally kept constant. These manipulations are highly unnatural and could potentially mask, if not remove, the subtle effect of non-photic cues such as temperature and their interactions with other cues like photoperiod (Dawson 2008; Paul et al. 2009). In the present study, we used a spring-like slow increase in both photoperiod and temperature (see Fig. S3.1). Despite this more realistic pattern of cue progression, we still did not find an effect of the temperature treatment on the onset of breeding (see above for potential explanations).

Our wild great tit population is currently laying too late in the season to match the peak in the abundance of their nestlings' food. This mistiming is the result of climate change that has induced a stronger shift in this food peak compared to the shift in laying date (Visser et al. 1998; Visser et al. 2006). As a consequence, there is now directional selection for earlier laying. As laying date is also heritable (Sheldon et al. 2003; Gienapp et al. 2006; Caro et al. 2009; Husby et al. 2011), natural selection will likely lead to micro-evolution. To estimate the rate of micro-evolution we need to understand where in the cascade underlying seasonal timing the genetic variation lies (Visser et al. 2010a). Our results strongly suggest that there is genetic variation in cue sensitivity (of either photoperiod or temperature) and we thus predict micro-evolution on cue sensitivity, which perhaps could restore the synchrony in phenology between the birds and their prey. This may well be a genetic shift in sensitivity in photoperiod rather than temperature as this will also lead to an advancement of timing. In addition, a change in the sensitivity to temperature will not only advance birds over the entire temperature range but might also alter the slope of their laying date versus temperature reaction norm. As selection is stronger in warmer years, where birds are deemed to be especially late compared to the food peak, this may be a requirement to fully restore the synchrony in phenology. The key question remains whether this rate of micro-evolution will be sufficient to match the rate of climate change (Visser 2008). Given the very limited evidence for climate change-induced micro-evolution (Gienapp et al. 2008), this seems not likely at this moment.

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Chapter 4

Sonja V. Schaper, Alistair Dawson, Peter J. Sharp, Phillip Gienapp, Samuel P. Caro and Marcel E. Visser

Increasing temperature, not mean temperature, is a cue for avian timing of reproduction

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Abstract

Timing of reproduction in temperate-zone birds is strongly correlated with spring temperature, with an earlier onset of breeding in warmer years. Females adjust their timing of egg laying between years to be synchronized with local food sources and thereby optimize reproductive output. However, climate change currently disrupts the link between predictive environmental cues and spring phenology. To investigate direct effects of temperature on the decision to lay and its genetic basis, we used pairs of great tits (*Parus major*) with known ancestry and exposed them to simulated spring scenarios in climate-controlled aviaries. In each of three years, we exposed birds to different patterns of changing temperature. We varied the timing of a temperature change, the daily temperature amplitude and the onset and speed of a seasonal temperature rise. We show that females fine-tune their laying in response to a seasonal increase in temperature, whereas mean temperature and daily temperature variation alone do not affect laying dates. Luteinizing hormone concentrations and gonadal growth in early spring were not influenced by temperature or temperature rise, possibly posing a constraint to an advancement of breeding. Similarities between sisters in their laying dates indicate genetic variation in cue sensitivity. These results refine our understanding of how changes in spring climate might affect the mismatch in avian timing and thereby population viability.

Introduction

The timing of life-cycle events, such as breeding and migration in birds, is a phenomenon that has intrigued biologists for decades. In the temperate zone, strong seasonal fluctuations in food availability determine a short period in spring suitable for reproduction in terms of energy and nutrient availability (Lack 1968; Perrins 1970; Verhulst and Tinbergen 1991). In this period, birds have to lay eggs and raise their offspring. After breeding, they require energy for moult, which has to be completed before conditions deteriorate. The timing of these connected life-cycle stages is crucial to maximize individual fitness (Wingfield and Kenagy 1991; Grieco et al. 2002; Both et al. 2004; Visser and Both 2005; Visser et al. 2006; Wingfield et al. 2008b). If the period of chick feeding matches the appropriate local food peak, both fledgling survival (Perrins 1965; van Noordwijk et al. 1995; Sheldon et al. 2003; Charmantier et al. 2008) and parental condition and survival (Thomas et al. 2001) are enhanced.

Because the timing of the food peak differs among years, temperate-zone birds, such as great tits (*Parus major*), show large annual variation in the onset of laying. This variation is largely due to phenotypic plasticity (Pigliucci 2001): females vary their laying dates according to climatic conditions. Great tits lay earlier, on average, in warm springs (e.g. Kluyver 1952; Perrins 1965; van Balen 1973; Schmidt 1984; Perrins and McCleery 1989), but individuals also differ consistently in their laying date relative to the yearly population mean (Nussey et al. 2005). This can be explained

by two possibly complementary hypotheses: first, females differ in their costs of egg production, allowing only high-quality birds to breed at the optimal time, and second, females differ in their use of environmental cues to assess the optimal laying date (Visser et al. 1998; Visser et al. 2010b).

Among these cues, increasing day length is considered an initial predictive cue (Silverin et al. 1993; Wingfield 1993; Dawson et al. 2001), acting either directly or via the entrainment of an endogenous circannual rhythm (Gwinner 1986; Gwinner 1996). Exposure to long days stimulates luteinizing and follicle-stimulating hormone secretion, gonadal development and sex steroid synthesis and release, which facilitates the start of breeding. However, supplementary cues (Wingfield and Kenagy 1991; Wingfield et al. 1992; Dawson 2008), such as temperature, provide information about local conditions and accelerate or delay reproductive development. Observations of tropical birds show a direct relationship between supplementary cues and the onset of breeding (Hau et al. 2000; Wikelski et al. 2000; Hau 2001), but this has seldom been experimentally investigated in temperate-zone birds.

Many tree species, and also arboreal caterpillars that passerine birds rely on in spring to feed their chicks (Perrins 1991; Naef-Daenzer et al. 2000; Visser et al. 2006), respond directly to temperature (van Asch et al. 2007). Field observations show a negative relationship between spring temperatures and mean laying date (Slagsvold 1976; Perrins and McCleery 1989; Crick et al. 1997b; McCleery and Perrins 1998; Sokolov 2000; Both et al. 2004; but cf. exceptions in Visser et al. 1998; Visser et al. 2003); however, they never exclude the possibility of indirect temperature effects via, for instance, spring phenology. Experiments comparing artificially heated or cooled nest boxes (Yom-Tov and Hilborn 1981; Nager and van Noordwijk 1992) failed to provide unambiguous evidence for an advancement in laying through warming. Most laboratory-based experiments employ a strong increase in photoperiod or compare constant and extreme temperatures (Storey and Nicholls 1982; Silverin and Viebke 1994; Silverin 1995; Wingfield et al. 1996; Wingfield et al. 1997; Wingfield et al. 2003), although not all do so (Suomalainen 1937; Perfito et al. 2005). Moreover, laying under controlled conditions is rare because of the difficulties of providing captive birds with an environment where they reproduce (but see Meijer et al. 1999; Lambrechts and Perret 2000; Caro et al. 2007). Visser et al. (2009) simulated temperature patterns recorded during years in which mean laying dates in the wild population of great tits differed by two weeks. They showed that females under simulated cold/late-year conditions delayed their reproduction compared to the group under warm/early-year conditions. In contrast, Visser et al. (2011a), using linear temperature increases differing only in mean temperature, found no distinction in laying dates. Apparently, a constant difference did not capture the temperature effect from the mimicked cold and warm years used by Visser et al. (2009).

Our aim was to unravel the characteristics of a seasonal temperature profile that affect laying dates of great tits. We used unique climate-controlled bird facilities to observe egg laying under controlled photoperiod and temperature conditions. Furthermore, we analyzed temperature effects on endocrine and gonadal development and postnuptial moult, encompassing a large part of the avian life cycle under simulated environmental conditions.

In 2008, we lowered ambient temperature at specific time periods while keeping a control group at a constant temperature. If mean temperature acts as a cue, then birds under constant warm conditions should lay first. Furthermore, if temperature cues become more important as the season proceeds, we expect birds under cold conditions late in spring to lay latest. In 2009, we investigated whether daily variation in temperature affects the onset of laying. Under natural conditions, the difference between daily maximum and minimum temperatures increases progressively during spring. We therefore evaluated the effect of a high or low 24 h temperature amplitude embedded within a high or low mean temperature. If birds respond to daily temperature changes, then females that experience high fluctuations around a warm mean should lay first. Furthermore, if minimum temperature, which restricts the development of invertebrate food (Partridge et al. 1994; Petavy et al. 2001), serves as a cue, then we expect birds experiencing the lowest temperatures to lay latest. In 2010, we simulated patterns of spring increases in temperature that differed in their timing, speed and maximum, as well as in the timing of a subsequent temperature rise just before laying. If birds use the increase in early spring as a cue, then birds exposed to an early and steep temperature increase should lay first. However, if birds fine-tune their laying by using the temperature rise immediately preceding laying, then we expect a (potentially additional) advancement.

When individuals vary their timing in response to a cue and this response has a genetic component (van der Jeugd and McCleery 2002; Sheldon et al. 2003; McCleery et al. 2004; Nussey et al. 2005), it is crucial to compare related individuals in controlled experiments. Here we present the outcome of three years of experiments on birds of known ancestry from a long-term monitored population.

Materials and Methods

Birds

We used 36 first-year breeding pairs of great tits in each of the three years. Birds were collected from a long-term study population at the Hoge Veluwe National Park (The Netherlands) and taken to captivity as nestlings in 2007-2009. Each year, 10 broods were selected from specifically early- or late-laying maternal lines: five early and five late broods (avoiding replacement broods) with known information about

the previous early- or late-laying history of the female herself and/or her mother and grandmother. All chicks were blood sampled when 3 days old and sexed (Griffiths et al. 1998) and extra-pair offspring were identified (Saladin et al. 2003) before brood choice. On day 10 after hatching, chicks were taken for hand-raising (Drent et al. 2003) to the Netherlands Institute of Ecology (Heteren), where they were weighed (chick weights) as a proxy for early environment condition.

We tested all fledglings for exploration behaviour in a novel environment as a measure of personality (Verbeek et al. 1994; Dingemanse et al. 2002). Afterward, they were transferred to open outdoor aviaries (2 x 4 x 2.5 m), where they were housed in groups. In December, breeding pairs were formed randomly, avoiding sib matings. In 2008 and 2010, early-laid females were paired with early-laid males and vice versa, while in 2009 all four possible combinations of pairs were formed to decouple sex-specific effects. On 1st December, the birds were placed in climate-controlled aviaries to breed in the next year.

Because of fatalities in the young birds from the Hoge Veluwe, we formed some pairs by using additional hand-raised birds with known laying dates. In 2008, five females and three males from an adjacent field site were used. In 2009 and 2010, birds hatched from eggs laid by captive great tits but cross-fostered and raised by wild parents until day 10, the start of hand-rearing, were used: eight females and four males in 2009, one female and nine males in 2010.

Aviaries

We kept the breeding pairs in 36 separate indoor aviaries (2 x 2 x 2.25 m) under a photoperiod that was adapted twice a week following the natural change in day length (for 52°N, increasing from 7.45L(light):16.15D(dark) at the winter solstice to 16.30L:7.30D at the summer solstice). Light sources were three high-frequency fluorescent light tubes, complemented with an 8-W bulb for an additional half-hour of dawn and dusk. A roof shaft (SolaTube), synchronized with the light schedule, opened to allow for supplementary daylight.

The birds were fed *ad libitum* with a constant daily amount of food (Visser et al. 2011a). Nesting material was provided from March onward. Birds could choose between two nest boxes, which we inspected for eggs from outside the aviary without disturbance.

Temperature treatments

Previous studies (Visser et al. 2009; Visser et al. 2011a) showed that birds vary their egg-laying behaviour with temperature in the absence of other supplementary cues. The study described here extends previous results to pinpoint which aspects of a temperature profile influence laying dates. The temperatures are well within

the ranges present in our study area over the past 10 years (maxima of 15°/14°C in December/January and minima of 6.5°/6°C in July/August, respectively). Each season, a different setup of four temperature treatments was used (Fig. 4.1, left), replicated in a regular design over nine aviaries.

Our aim in 2008 was to examine at which springtime period females were most sensitive to temperature changes. All four treatment groups were kept at a constant temperature of 15°C from December onward. In three groups, this temperature was lowered to 7°C for a month, in February, March, or April, before it was increased to 15°C again, except for the latest cold period, which was maintained until the female initiated laying under cold conditions (Fig. 4.1A). When a female started moulting, that is, shed the first primary feather, temperature was increased to 20°C in each aviary individually.

In 2009, our experiment focused on effects of daily fluctuation in temperature. If temperatures were not perceived as absolute values but rather as daily variation, this would allow a bird to measure the progress of spring via the experienced temperature amplitude over a day. To investigate this, we exposed birds to one of four temperature treatments, each composed of a high or low mean temperature with either a high or low day-night amplitude (Fig. 4.1D). The warm treatments fluctuated around 14°C (11°-17°C for high amplitude, 13°-15°C for low amplitude) and the cold treatments around 8°C (5°-11°C for high amplitude, 7°-9°C for low amplitude). The minimum was reached at 3:00 a.m. There was no seasonal pattern. When females started moulting, the temperature was increased to 20°C.

The 2010 setup combined two consecutive temperature rises, one during early gonadal development and the other shortly before breeding. We initially kept all birds at 6°C. On 8th February (January day 39, 1=1st January), the temperature was increased rapidly for two groups from 6° to 16°C over the course of 2 weeks and then maintained at 16°C for 3 or 5 weeks (Fig. 4.1G). Starting on 15th or 29th March, the temperature was increased to 20°C over 1 week and stayed high during egg laying and moult. Starting on 22nd February (January day 53), the other two groups were exposed to a more gradual increase in temperature, from 6° to 11°C over a course of 2 weeks, thus experiencing a lower increase rate. These groups were then held at 11°C for 1 or 3 weeks. Starting on 15th or 29th March, temperature was increased to 15°C for egg laying and moult (Fig. 4.1G). Superimposed on the temperature profiles was a day-night rhythm of ±1°C.

Data collection

We checked nest boxes daily for eggs, which were collected and replaced by eggs taken from nests of wild great tits used in cross-fostering experiments. Complete nests were removed after 5 days of incubation, after which some females rebuilt nests

and initiated new clutches. The day the first egg was found is referred to as the laying date and the day the last egg of the last clutch was laid is referred to as the date of termination of reproduction.

A 100-ml blood sample was taken monthly from the jugular vein. Samples were kept on ice until centrifugation, and plasma was separated and stored at -80°C. Plasma luteinizing hormone (LH) concentrations were determined with a chicken LH radioimmunoassay (Sharp et al. 1987) validated for use in blue tits (Caro et al. 2006). The assay reaction volume was 60 µl, comprising 20 µl of plasma sample or standard, 20 µl of primary antibody (rabbit anti-chicken LH) and 20 µl of ^{125}I-labeled LH. The primary antibody was precipitated to separate free and bound ^{125}I label by use of 20 µl of donkey anti-rabbit precipitating serum and 20 µl of non-immune rabbit serum. All samples from each year were measured in a single assay, in duplicate. The intra-assay coefficient of variation was 6.4% for a high-value pool and 8.1% for a low-value pool; the minimum detectable dose was 0.15 ng/ml.

In alternation with blood sampling, we performed a monthly laparotomy to measure gonadal development. Males were assessed from January to July and females up to April in order not to interfere with laying. However, in 2009 females were not laparotomized in April, with no effect on the onset of laying, and in 2010 we did no laparotomy in January, as little variation in gonad sizes had been shown in previous years. Birds were unilaterally laparotomized under isoflurane anaesthesia (Forene, Abbott, Hoofddorp, Netherlands). Left-testis length and width and the diameter of the largest follicle in the ovary were measured to the nearest 0.1 mm with an ocular scale. Testis volume was calculated as $V=4/3\pi a^2 b$, where a is half the width and b is half the length; follicle volume was calculated as $V=4/3\pi a^3$, where a is half the width.

We recorded the moult of the primary wing feathers biweekly in 2008 and weekly in 2009 and 2010. The moult score was calculated following Dawson and Newton (2004), with great tit–specific parameters from Dawson (2005b). For each individual, date was linearly regressed against moult score. The onset (intercept) and speed (slope) of moult allowed us to calculate moult duration.

Statistical analyses

The onset or termination of reproduction can be viewed as a "time to event" and analyzed with mixed-effects Cox proportional hazards models (Cox 1972). The probability that an animal will start laying on a particular day is a function of an unspecified baseline hazard (Kalbfleisch and Prentice 2002) multiplied by a number of explanatory variables, which include fixed as well as time-dependent variables. This approach has been applied to the analysis of timing of reproduction and migration of wild populations (Gienapp et al. 2005; Bauer et al. 2008; Gienapp et al. 2010).

The advantage of this method over regression of individual observations against a temperature measure is that there is no need to specify a fixed time period of interest. Instead, we used an iterative procedure based on a linear predictor for the calculation of a temperature variable (λ) that incorporates the current as well as earlier temperatures experienced by the organisms (for details, see Gienapp et al. 2005). A weighting factor (α) assesses the relative importance of current versus earlier temperatures: if this factor is large, the model places a high weight on the most recent temperature (the temperature variable resembles the mean of that particular day), while if it is small, previous temperatures are given more importance, which implies a longer "memory" of temperature conditions. Values for the weighting factor ranged from 0.01 to 0.2 in increments of 0.01. In the model-reduction process (based on a partial maximum likelihood method), the best linear temperature predictor λ was recalculated in each reduction step. The mean December temperature of individual aviaries was used as a starting value, and daily mean temperatures modified by the weighting factor were used as time-dependent variables. Use of minimum temperatures did not give qualitatively different results. Statistical significance was tested with likelihood ratio tests (Therneau and Grambsch 2000). We used the coxme (including random effects) and coxph procedures from package kinship in R 2.10.0 (R Development Core Team 2009).

Temperature was coded either as a four-level treatment or as time-dependent realized temperatures (integrated by α and therefore including within-treatment variation over time). For each year, a complex model incorporated female family as a random factor, temperature, chick weight (measure for early-life condition), personality (responsiveness to environmental variability and social stimuli) and date of birth (date of the mother's first egg of the clutch from which that bird originated, i.e. early- and late-laid birds). Furthermore, the interaction between female date of birth and temperature was added. The time-dependent interaction between photoperiod and temperature was included in the model for onset of laying. Because day length is linearly related to date, it was impossible to include as a main effect. In 2009, the genetic composition of the pair (e.g. early-laid female-late-laid male) was added. In addition, models that used only female or male family as explanatory variables while controlling for temperature treatment were used to assess differences in laying behaviour between families.

Gonadal development was analyzed in mixed models (procedure lmer, package lme4 in R 2.10.0), with individual as a random effect. Data on gonadal maturation (January-April) was log transformed to account for exponential growth. Fixed effects were month, tarsus length as a measure of body size, temperature treatment, date of birth and the interaction between temperature treatment and date of birth. Male gonadal regression did not follow a negative exponential pattern, and log transformation did not facilitate a better model fit. Testis volume was therefore analyzed separately for

May-July. As tarsus length was not correlated to gonadal growth, it was excluded from the analyses of gonadal regression. Family was fitted as a random effect.

Luteinizing hormone levels were log transformed and analyzed over the whole season in mixed models using individual and family as nested random effects. Fixed effects were month as a factor, temperature treatment, date of birth, personality and date of birth of the mate as well as the interactions between temperature treatment and month and temperature treatment and date of birth.

Onset and duration of moult were analyzed in mixed models using family as a random effect. First, laying/non-laying birds were compared, showing that birds that skip reproduction mostly start moult earlier. A subsequent analysis included only laying pairs and used date of termination of reproduction, temperature treatment and date of birth as fixed effects.

Results

Onset of reproduction

In 2008, when the effect of cold periods in different spring months was tested, 24 out of 36 pairs initiated at least one clutch, starting on 17th April. Temperature treatments affected the onset of laying differently for early- and late-laid birds (early-laid birds had a mother that laid early). Cold conditions in February or March, followed by a temperature rise, made early-laid females lay significantly earlier than early-laid females in constant warm conditions or early-laid females that experienced a cold period in April with no subsequent temperature rise. In contrast, cold conditions in February or March, followed by a temperature rise, made late-laid females lay significantly later (Table 4.1a; Fig. 4.1B,C). In the model using realized temperatures as a time-dependent variable, the small value of the weighting factor (α=0.04) indicated a long-term temperature integration to be appropriate. This long-term pattern especially influenced the laying decision of late-laid females: overall cold conditions induced an advance and warm conditions a delay. In contrast, early-laid females did not change their laying date in response to long-term temperature (Table 4.1b).

In 2009, when the effect of daily temperature variation was tested, 27 pairs initiated at least one clutch, starting on 15th April. Neither temperature treatment nor realized temperature influenced the onset of laying (Table 4.1; Fig. 4.1E,F). Note that in this setup, daily means did not distinguish between high- and low-daily-amplitude treatments, except for variation between aviaries. An analysis comparing minimum temperatures also failed to show any temperature effect on laying date (data not presented). The onset of laying in early- and late-laid females was not influenced by whether they were paired with males from either late- or early-laying families (pair composition; Table 4.1).

In 2010, when the effect of temperature-increase patterns in early and late spring was tested, 18 pairs initiated at least one clutch, starting on 23rd April, about 2 weeks after the last increase in temperature. The onset of reproduction was determined by the temperature rise shortly before laying: an earlier rise in late spring led to earlier laying, independent of the temperature pattern in early spring (Table 4.1a; Fig. 4.1H,I). The weighting factor for the best time-dependent temperature variable was fairly large (α=0.14), indicating that the laying date in this year was based on recently experienced temperatures. Overall colder spring conditions advanced the start of laying (Table 4.1b).

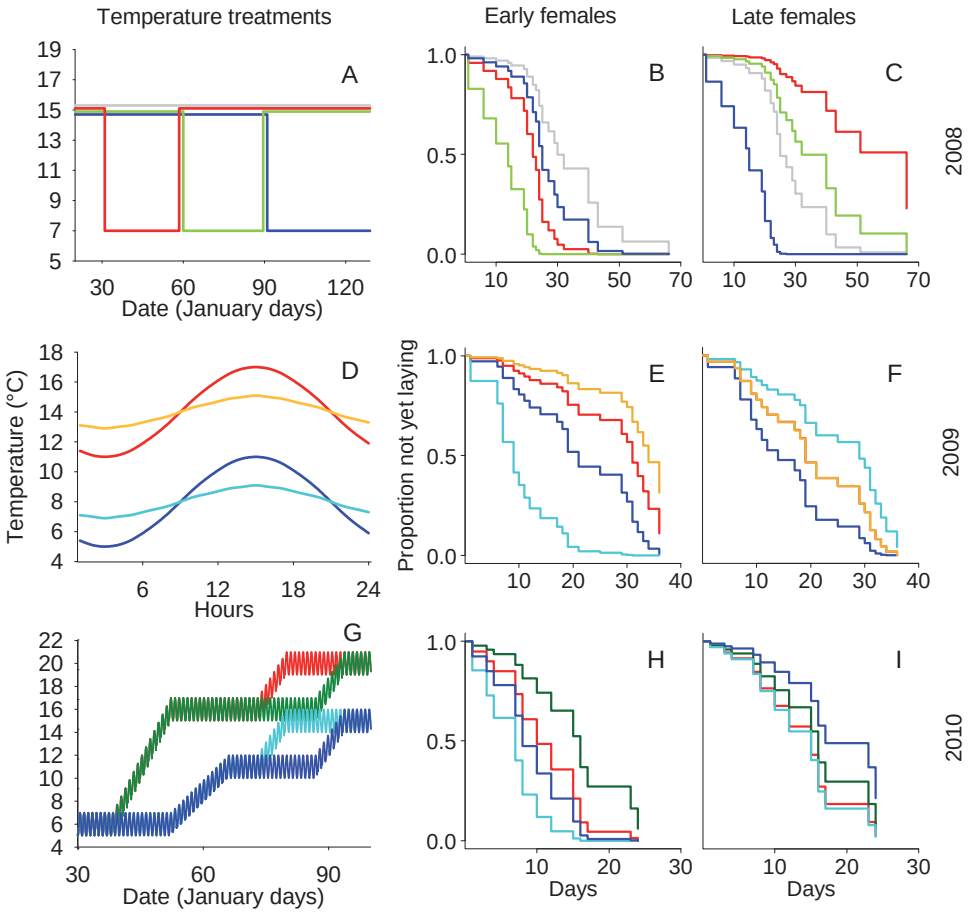

Figure 4.1: Temperature treatments (left) and their effect on the onset of laying for females from early (middle) and late families (right) in climate-controlled aviaries in 2008 (A-C), 2009 (D-F), and 2010 (G-I). Colours of the temperature patterns on the left identify the treatments in all subsequent graphs. For a description of the treatments see "Materials and Methods." Note that in 2009 the temperature pattern did not vary seasonally. B, C, E, F, H, I, Survival graphs showing the outcome of the proportional hazards model in Table 4.1 a. Lines show the proportion of females per treatment that are not yet laying in relation to the first laying date of the year. The middle panels show the earliest-laying family of each year (mother's laying dates: 7th April, 2008; 3rd April, 2009; 8th April, 2010) and the right-hand panels the latest-laying family (mother's laying dates: 20th April, 2008; 26th April, 2009; 18th April, 2010). Days represent days after the first laying date: 17th April, 2008, 15th April, 2009, and 23rd April, 2010. A fast-descending line represents a group that starts laying early. Note that in F the red and yellow lines are superimposed.

Table 4.1: Effects of temperature treatment, or realized temperature, and individual traits on the onset of reproduction. We tested how the onset of reproduction 2008-2010 was affected by temperature treatments (a) or realized temperatures (b) and individual traits. The best weighting factor α is given below the table for each year. Female family was fitted as a random effect. Statistics are given for the point of exclusion from the model. In case of significant interactions, statistics for the components are given in presence of the interaction. Therefore statistics for a continuous variable cannot be provided for an interaction of this variable and a factor.

	2008			2009			2010		
	χ^2	df	P	χ^2	df	P	χ^2	df	P
a) Temperature treatment and individual traits									
Temperature treatment	23.80	3	**<0.001**	4.52	3	0.21	15.89	3	**0.001**
Laying date of female's mother				0.67	1	0.41	3.81	1	0.051
Treatment * Laying date of female's mother	24.67	3	**<0.001**	2.04	3	0.15	1.60	3	0.21
Laying date of male's mother	1.79	1	0.17	0.01	1	0.91	10.62	1	**0.003**
Female personality	12.28	1	**0.005**	3.36	1	0.081	10.43	1	**0.004**
Female chick weight	5.06	1	**0.003**	0.81	1	0.38	2.37	1	0.12
Male personality	1.76	1	**0.010**	0.75	1	0.41	12.68	1	**0.003**
Male chick weight	3.46	1	0.081	0.12	1	0.72	7.84	1	**0.010**
Pair composition				6.88	3	0.076			
b) Realized temperature and individual traits									
Realized temperature	2.27	1	0.13	0.57	1	0.57	5.46	1	**0.012**
Laying date of female's mother	5.48	1	**0.019**	0.67	1	0.41	0.88	1	0.37
Temperature * Laying date of female's mother	6.11	1	**0.010**	0.21	1	0.65	0.52	1	0.40
Temperature * photoperiod	0.17	1	0.68	3.82	1	0.051	1.40	1	0.25
Laying date of male's mother	0.25	1	0.62	1.28	1	0.26	0.13	1	0.71
Female personality	0.46	1	0.50	3.36	1	0.081	0.73	1	0.36
Female chick weight	0.48	1	0.49	1.80	1	0.19	5.17	1	**0.015**
Male personality	0.01	1	0.93	3.10	1	0.11	0.62	1	0.57
Male chick weight	0.24	1	0.63	0.11	1	0.74	<0.01	1	0.96
Pair composition				6.88	3	0.076			
Best weighting factor α		0.04			0.01			0.14	

To test for genetic effects on timing, we compared laying dates, after controlling for temperature treatment, within and across families. In 2009 and 2010, females from the same family had similar laying dates (female family 2009: χ^2_{14}=26.84, P=0.02, 2010: χ^2_8=21.98, P=0.005), while in 2008 and 2010 there was a similarity of females mated to brothers (male family 2008: χ^2_{11}=34.39, P<0.001, 2010: χ^2_{10}=30.03, P<0.001).

Termination of reproduction

Data on the termination of reproduction are presented in Appendix 4.1, available online. In summary, only the temperature development late in spring played a role in the termination of laying. Comparably to the onset of reproduction, early- and late-laid females reacted differently to temperature cues. In all years there was a consistent resemblance between sisters, suggesting a strong genetic component (2008: χ^2_{10}=30.25, P<0.001, 2009: χ^2_{14}=29.15, P=0.010, 2010: χ^2_8=16.52, P=0.036).

Additional effects of individual characteristics

In 2008, high novelty-seeking behaviour and higher female chick weights induced earlier laying in adults, while in 2010 laying was delayed by high novelty-seeking behaviour and also when females had a partner that was an early-laid or light chick (Table 4.1a). In models using realized temperature, females that had been heavier chicks laid later in 2010 (Table 4.1b). Because of inconsistency, these observations are deemed not to be biologically meaningful.

Onset and duration of moult

Moult data are presented in Appendix 4.2. In most years, non-laying birds started moult significantly earlier than laying pairs. In general, the later a bird stopped reproducing, the later it started moulting. There was no effect of temperature treatment on the onset of moult. Some birds experiencing a cold spring period in 2008 moulted significantly faster, even though moult took place under constant warm conditions.

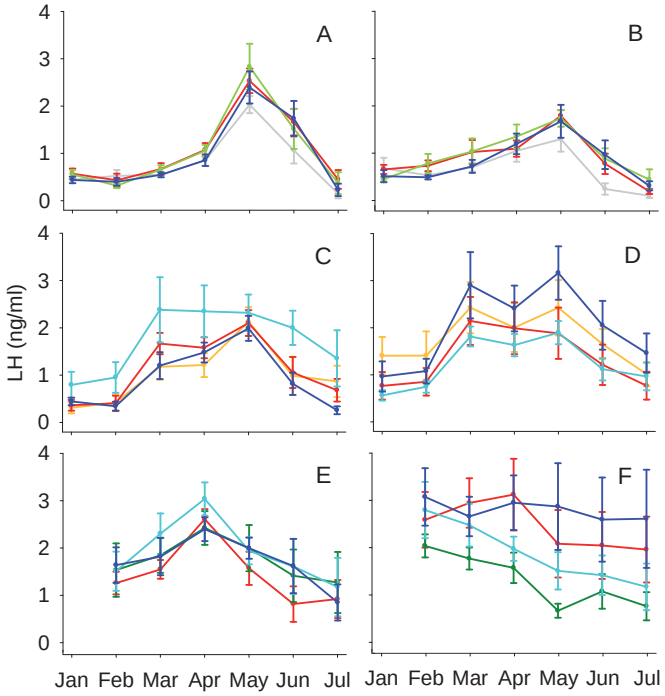

Figure 4.2: Changes in the concentration of plasma luteinizing hormone (LH) in females (left) and males (right) for the years 2008 (A, B), 2009 (C, D), and 2010 (E, F). Birds were exposed to temperature treatments shown in Figure 4.1, with the same colour coding. Means ± standard errors are shown.

Luteinizing hormone (LH)

Female LH plasma concentrations increased over spring in all years, peaking in April/May (Fig. 4.2A,C,E). The same distinct seasonal peak was found in males in 2008. However, males in 2009 had a wider peak, while in 2010 males reached maximum concentrations in early spring (Fig. 4.2B,D,F). Temperature treatment did not influence LH concentrations, except in 2009, when females experiencing a narrow temperature variation around a low mean tended to have elevated levels (Table 4.2). Temperature treatments affected early- and late-laid males differently in 2009: early-laid males experiencing warm temperatures and a wide variation had lower LH concentrations than late-laid males in all treatments (Table 4.2). In 2009, low novelty-seeking behaviour was correlated with low LH concentrations (Table 4.2), also observable as a trend in males of 2008.

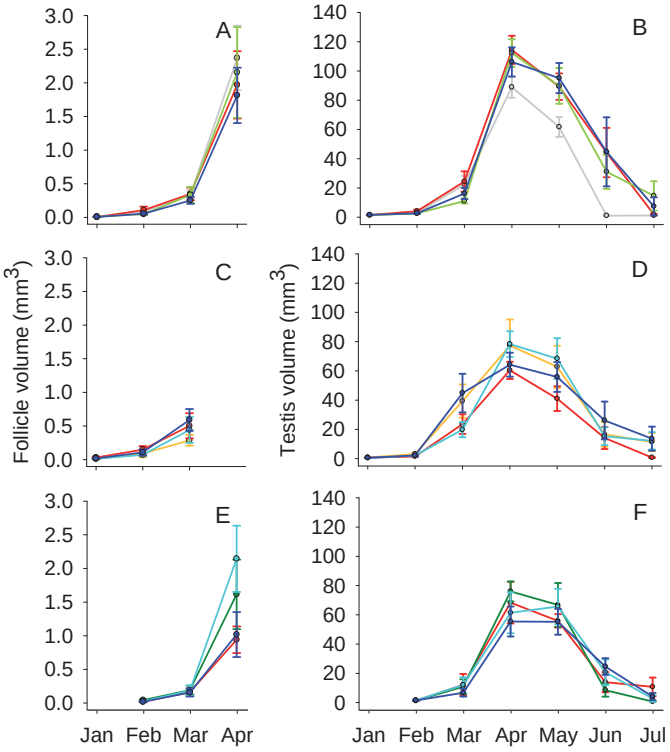

Figure 4.3: Growth of the largest ovarian follicle in females (left) and the left testis in males (right) for years 2008 (A, B), 2009 (C, D), and 2010 (E, F). Birds were exposed to temperature treatments shown in Figure 4.1, with the same colour coding. Means ± standard errors are shown.

Gonadal development

The largest ovarian follicles grew exponentially over time, but temperature treatments had no effect on their size in any year (Table 4.3a; Fig. 4.3). In 2010, early-laid females grew their follicles faster than late-laid females (Table 4.3a). Testis sizes followed a typical pattern of steep recrudescence, peaking in April, and subsequent regression. Testis maturation was independent of temperature treatment in any given year (Table 4.3b; Fig. 4.3). However, in 2008, gonadal regression was delayed in males that experienced a cold period compared to that in males under a constant warm temperature (Table 4.3c; Fig. 4.3B). In addition, in some months the rate of testis regression differed between early- and late-laid males (Table 4.3), but not in a consistent pattern.

Table 4.2: Effects of temperature treatment and individual traits on luteinizing hormone (LH) development. Hormonal development between January and July 2008-2010 (not containing January in 2010) in females (a) and males (b) was log-transformed. Individual and family were fitted as random effects. Statistics are given for the point of exclusion from the model. In case of significant interactions, statistics for the components are given in presence of the interaction. Therefore statistics for a continuous variable cannot be provided for an interaction of this variable and a factor.

	2008			2009			2010		
	χ^2	df	P	χ^2	df	P	χ^2	df	P
a) LH (log) females									
Temperature treatment	3.18	3	0.36	7.64	3	0.054	1.61	3	0.66
Month	219.3	6	**<0.001**	165.2	6	**<0.001**	110.9	5	**<0.001**
Treatment * Month	15.11	3	0.65	19.76	3	0.35	10.21	3	0.81
Laying date of female's mother	0.70	1	0.40	<0.01	1	1	3.35	1	0.067
Treatment * Laying date of female's mother	0.95	3	0.81	1.79	3	0.62	0.56	3	0.91
Laying date of male's mother	1.19	1	0.27	0.06	1	0.81	0.72	1	0.40
Personality female	0.76	1	0.38	5.18	1	**0.023**	0.40	1	0.53
b) LH (log) males									
Temperature treatment	4.47	3	0.21	18.3	3	**<0.001**	1.71	3	0.64
Month	155.2	6	**<0.001**	171.0	6	**<0.001**	85.32	5	**<0.001**
Treatment * Month	25.07	3	0.12	22.48	3	0.21	9.59	3	0.84
Laying date of male's mother	<0.01	1	1				<0.01	1	1
Treatment * Laying date of male's mother	1.22	3	0.75	15.40	3	**0.002**	1.45	3	0.69
Laying date of female's mother	0.15	1	0.70	0.77	1	0.38	0.36	1	0.55
Personality male	3.63	1	0.057	4.05	1	**0.044**	1.15	1	0.28

Table 4.3: Effects of temperature treatment and individual traits on gonadal development. Gonadal recrudescence for females (a) and males (b) between January and April 2008-2010 (not containing April 2009 for females and January 2010 for both sexes) was log-transformed. Testicular volume in May, June and July was analyzed in separate models (c, d, e). Individual (in case of gonadal recrudescence) and family was fitted as a random effect. Statistics are given for the point of exclusion from the model. In case of significant interactions, statistics for the components are given in presence of the interaction. Therefore statistics for a continuous variable cannot be provided for an interaction of this variable and a factor.

	2008			2009			2010		
	χ^2	df	P	χ^2	df	P	χ^2	df	P
a) Follicle volume January-April (log)									
Temperature treatment	0.50	3	0.92	2.60	3	0.46	3.65	3	0.30
Laying date of female's mother	2.36	1	0.12	0.26	1	0.61	9.22	1	**0.002**
Treatment*Laying date of female's mother	4.19	3	0.24	2.51	3	0.47	1.46	3	0.69
Tarsus female	1.03	1	0.31	1.55	1	0.21	0.18	1	0.67
Month	261.2	1	**<0.001**	97.71	1	**<0.001**	123.2	1	**<0.001**
b) Testis volume January-April (log)									
Temperature treatment	2.35	3	0.50	2.61	3	0.46	0.44	3	0.93
Laying date of male's mother	0.01	1	0.94	<0.01	1	1	<0.01	1	1
Treatment*Laying date of male's mother	0.80	3	0.85	3.11	3	0.38	0.25	3	0.97
Tarsus female	3.21	1	0.073	0.66	1	0.42	2.66	1	0.10
Month	317.3	1	**<0.001**	268.4	1	**<0.001**	161.8	1	**<0.001**
c) Testis volume May									
Temperature treatment	9.41	3	**0.024**	5.05	3	0.17	1.10	3	0.78
Laying date of male's mother	0.52	1	0.47	5.31	1	**0.021**	0.06	1	0.80
Treatment*Laying date of male's mother	1.76	3	0.62	4.88	3	0.18	0.37	3	0.95
d) Testis volume June									
Temperature treatment	3.71	3	0.29	9.28	3	**0.026**	3.57	3	0.31
Laying date of male's mother	4.06	1	**0.044**				1.11	1	0.29
Treatment*Laying date of male's mother	6.70	3	0.082	8.45	3	**0.038**	2.16	3	0.54
e) Testis volume July									
Temperature treatment	5.75	3	0.12	5.04	3	0.17	4.03	3	0.26
Laying date of male's mother	2.38	1	0.12	<0.01	1	1	6.96	1	**0.008**
Treatment*Laying date of male's mother	3.00	3	0.39	4.56	3	0.21	2.88	3	0.41

Discussion

Our studies are among the first to investigate how spring temperature patterns affect laying dates under controlled conditions. We show clearly that, despite previous assumptions, warm temperatures alone do not accelerate the onset of laying in great tits. In 2008, females that experienced a constant warm spring did not, on average, lay earlier than birds exposed to a cold period. In 2009, irrespective of the daily amplitude around the mean, females kept under a high average temperature did not lay earlier than females kept under a low temperature. In 2010, females that experienced a fast and warm early spring (green and red lines in Fig. 4.1G) did not lay earlier overall than females that experienced a slow and colder spring (blue lines in Fig. 4.1G). The lack of an advancing effect of higher mean temperature is in accordance with the results of Visser et al. (2011a), who did not find a difference in laying dates between females from cold and warm treatments that experienced a similar progressive increase in temperature. As it is well known that birds in the wild (e.g. Cresswell and McCleery 2003; Both et al. 2004) and in captivity (Visser et al. 2009) do lay earlier under natural temperature patterns of warm years, the key question is to which component of the patterns of change birds respond.

Our results clearly suggest that the relevant information resides in the periods of increasing temperature. In 2008, exposure to a temperature increase at the end of February or March advanced the onset of laying in genetic early layers and delayed it in genetic late layers, compared to that in females in other treatments. This shows that birds are sensitive to an increase in temperature well in advance of laying; this is also indicated by the best integrated temperature variable, which shows a long-term integration of temperature to be relevant for the laying decision. In 2010, a temperature increase close to the laying period advanced laying, independently of the temperature pattern in early spring. Similar to 2008, an overall warmer spring even delayed laying in 2010. The relevance of more recent temperatures was also reflected in the choice of the best integrated-temperature variable. The importance of a temperature rise close to laying resembles findings by Meijer et al. (1999), who observed that pairs of starlings (*Sturnus vulgaris*) kept in groups in climate chambers started laying about a week after a 5°C temperature increase at different dates in April.

These observations show that the weight given to specific temperature cues changes over spring and that these cues are interpreted differently by birds with different genetic backgrounds. There is no universal answer to the question of which spring-period changes in temperature have the largest effect on the laying date, as this depends on the nature of the temperature pattern. Results from 2008 and 2010 show that around March, an increase in spring temperatures to a moderate level advances the onset of laying, especially for birds from early-laying families. A moderate increase at this time, not rising too high, might be recognized as an indication of a long season,

with conditions adequate to allow for more than one breeding attempt. Early-laying females could therefore try to raise their first clutch in advance of the food peak, increasing their chances of raising a second brood as well (Crick et al. 1993; Verboven and Verhulst 1996). We know that in our population more pairs produce a second brood in colder years (Husby et al. 2009); however, patterns of temperature increase have not been assessed in this respect yet. Late-laying females also respond to a temperature increase around March but delay their reproduction. This could mean that they try to synchronize their only breeding attempt with the later-anticipated food peak. In 2010, a second temperature increase close to the laying period had an advancing influence on the onset of laying in both early- and late-laid females. In combination, these results demonstrate that birds that experience a temperature rise in early spring up to moderate temperatures lay early if they are from an early-laying family but that this genetic disposition can be overruled by a temperature increase close to laying, which induces early laying in all birds. It seems plausible that temperature cues can affect different developmental stages and that the implication of such cues, as well as the intensity of a behavioural response, changes over time (Wingfield 2008b).

In addition, we can exclude the possibility that birds integrate temperature cues for the initiation of laying as the experienced daily temperature range. Furthermore, a clear difference in minimum night temperature, an important factor restricting invertebrate development, did not affect laying dates in 2009.

The use of siblings allowed us to investigate genetic or early environmental effects on the timing of reproduction. In two years, sisters resembled each other in their laying date more than did unrelated females, which is consistent with an earlier study showing that temperature sensitivity in great tits depends on genotype (Visser et al. 2011a). In addition, laying dates in 2009 were closely correlated with laying dates recorded one year later by the same pairs in open aviaries (Schaper et al. 2011). Visser et al. (2009) also found a high repeatability in the distribution of laying dates of females breeding both in climate-controlled aviaries and in the wild. This consistency points to a similarity in the physiological setup of related females that leads to a certain timing of laying. That timing of reproduction has a heritable component is known for a number of species (Prendergast et al. 2004; Brommer et al. 2005; Nussey et al. 2005; Gienapp et al. 2006). If a large part of the response mechanism to environmental cues is determined by genetic differences, then the maintenance of diversity in these mechanisms indicates that they do equally well over a large number of years. Adaptations in the way cues are both perceived and physiologically transduced into behavioural responses should be further investigated.

In 2009, "mixed pairs," for example, early-family female - late-family male, were formed to decouple genetic dispositions. In that year, we found no support for indirect genetic effects (Brommer and Rattiste 2008; Visser et al. 2011a), that is, no male family effect and no clear influence of the pair composition.

Further, we cannot conclude that females that experienced good conditions as nestlings show carryover effects and lay early. In addition, even though social cues are certainly an important component of the laying decision (Helm et al. 2006), it is not apparent that males with a high personality score, which are more active singers (Naguib et al. 2010), induce their mates to lay early.

The termination of reproduction determines the earliest possible time to start moult and thus relates to energy partitioning and condition over winter. As temperature is indicative of the rate at which prey availability declines, it should affect the decision to stop laying, which we confirmed here. We found a consistent resemblance between sisters in the termination of laying in all years, possibly showing genetic differences in temperature sensitivity between early- and late-laying families, as these varied in termination dates in response to temperature. These intriguing results support similar findings by Visser et al. (2011a).

The timing of the rise in plasma luteinizing hormone (LH) concentration and gonadal maturation did not differ between birds that were exposed to diverse spring temperature patterns. This confirms that early stages of the reproductive cycle are not fine-tuned by temperature cues (Caro and Visser 2009). Despite a number of well-designed experiments, there is still conflicting evidence about temperature effects on reproductive physiology (e.g. Suomalainen 1937; Storey and Nicholls 1982; Silverin and Viebke 1994; Wingfield et al. 1996; Wingfield et al. 1997; Wingfield et al. 2003; Dawson 2005a; Perfito et al. 2005; Caro et al. 2009). At temperate latitudes, the timing of gonadal growth seems to be predominantly controlled by photoperiod. Therefore, in great tits a substantial advancement of laying due to benign spring conditions could be constrained by the functionality of the female reproductive system, setting an earliest possible laying date relative to the lengthening photoperiod. However, because the decision to lay itself is fine-tuned by the rise in temperature over the season, this points toward an unknown neuroendocrine mechanism, not reflected in gonadal development, that mediates the integration of temperature cues at the time of gonadal development and is involved in the laying decision. Because wild birds are exposed to a suite of interrelated cues, gonadal development is possibly fine-tuned by non-temperature cues.

Testis regression overall was not affected by temperature. However, it was advanced by a constant warm spring in 2008, even though the temperature difference of 8°C was small relative to that in other studies. That constant warm temperatures induce early regression (Silverin and Viebke 1994; Dawson 2005a; Silverin et al. 2008; Visser et al. 2011a) and that very low temperatures delay it (Jones 1986) are well documented, but possible mechanisms are still debated. High concentrations of prolactin are associated with gonadal regression and the start of moult (Dawson and Sharp 1998), and some studies have suggested that high temperatures may enhance prolactin secretion (Maney et al. 1999; Gahali et al. 2001). However, recent studies have shown no evidence

for this (Dawson and Sharp 2010; Visser et al. 2011a). Why differences in the timing of regression were observed only in 2008 is unclear, but constant temperatures might be a reason. Remarkably, even a cold period in February/March prolonged testis function, suggesting again the involvement of an elusive neuroendocrine signal that transduces temperature information toward later life-cycle stages.

Temperatures experienced in spring, as well as moderate temperature differences during the moulting period itself, do not play a relevant role in the timing of postnuptial moult in great tits. In contrast, our results underline the importance of the termination of reproduction in influencing later life-cycle stages.

Concluding remarks

Experiments on direct temperature effects on the timing of avian reproduction have produced ambiguous results, reflecting the complexity of the decision-making process. Our results show that an overall warmer spring by no means leads to an earlier onset of breeding but that it is rather the temperature increase that is used as a cue by female great tits to time their onset of laying. The mechanisms involved remain to be established. We therefore encourage further experiments under controlled conditions that take into account temperature variation, but we also encourage a re-evaluation of temperature patterns preceding the breeding season and possible correlations with laying dates in long-term-studied wild populations.

Our results further indicate that birds from different genetic backgrounds react differently to temperature cues in early and late spring. It has yet to be determined in what aspects these families differ. Environmental information that produces plasticity in laying has to be received, processed and integrated into a decision, a process mediated by the neuroendocrine system (Ball and Balthazart 2002; Lessells 2008; Lyon et al. 2008). The variation on which selection can act has to be genetic variation in (a combination of) underlying mechanisms (Wingfield et al. 2008b; Bourgault et al. 2010; Visser et al. 2010a). We still know too little about how and where temperature cues are integrated, in contrast to photoperiodic cues (Sharp 2005). We found no evidence that early stages of reproductive development were affected by spring temperature, which indicates that a so-far-unconsidered pathway, such as the hypothalamo-pituitary-thyroid axis (Wada 1993; Chastel et al. 2003; Wingfield et al. 2003), or a synergism involving multiple signals could be responsible for conveying the information that ultimately induces laying. However, to elicit an organized physiological response, supplementary cues, such as temperature patterns, presumably have to converge on the gonadotropin-releasing hormone system (Ball 1993; Hau 2001).

Climate change is affecting living systems in complex ways (Parmesan and Yohe 2003), and studies on phenology become increasingly relevant in this framework (Penuelas

and Filella 2001), as the initiation of life-history traits and the overall development of biological systems are highly temperature dependent (Parmesan and Yohe 2003; Root et al. 2003). In our study system, the relationship between food availability in spring and environmental cues used for timing of reproduction has shifted as a result of climate change, and great tits, as well as other small passerines, appear to be unable to compensate for this mismatch by adjusting their time of breeding (Visser et al. 1998; Both et al. 2004; Coppack and Pulido 2004). Our experiments offer insights into the scope of individual variation in response to temperature cues as well as into the genetic basis underlying it, which is crucial for natural selection to restore the synchrony of timing of reproduction with the altered phenology of environmental conditions.

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Chapter 5

Sonja V. Schaper, Alistair Dawson, Peter J. Sharp, Samuel P. Caro
and Marcel E. Visser

Individual variation in avian reproductive physiology does not reliably predict variation in laying date

Submitted manuscript

Abstract

Most animals reproduce seasonally. They time their reproduction in response to environmental cues, like increasing photoperiod and temperature, which are predictive for the time of high food availability. Although individuals of a population use the same cues, they vary in their onset of reproduction, with some animals reproducing consistently early or late. In avian research, timing of reproduction often refers to the laying date of the first egg, which is a key determinant of fitness. Experiments measuring temporal patterns of reproductive hormone concentrations or gonadal size under controlled conditions in response to a cue commonly assume that these proxies are indicative of the timing of egg laying. This assumption often remains untested, with few studies reporting both reproductive development and the onset of laying. We kept in total 144 pairs of great tits (*Parus major*) in separate climate-controlled aviaries over 4 years to correlate pre-breeding plasma luteinizing hormone (LH), prolactin (PRL) and gonadal growth with the timing of laying. Individuals varied consistently in hormone concentrations over spring, but this was not directly related to the timing of gonadal growth, nor with the laying date of the first egg. The timing of gonadal development in both sexes was similarly not correlated with the timing of laying. This demonstrates the female's ability to adjust the onset of laying to environmental conditions irrespective of substantial differences in pre-laying development. We conclude that stages of reproductive development are regulated by different cues, and therefore egg laying dates need to be studied to measure the influences of environmental cues on timing of seasonal reproduction.

Introduction

Seasonal timing of reproduction is a key life-history trait with a large impact on reproductive output. A mismatch between reproduction and seasonal high food abundance leads to fewer surviving and lower quality offspring, or lower winter survival of the parents (Perrins 1965; van Noordwijk et al. 1995; Thomas et al. 2001; Sheldon et al. 2003; Visser et al. 2006; Charmantier et al. 2008). In avian research, timing of reproduction often refers to the laying date of the first egg in spring (Visser et al. 2010a). However, the initiation of gonadal growth and the underlying activation of the reproductive endocrine system is also part of the timing mechanism (Hahn 1998; Hau et al. 2000; Dawson 2005a; Perfito et al. 2005; Caro et al. 2006; Moore et al. 2006; Silverin et al. 2008; Stevenson et al. 2008; Liedvogel et al. 2009). This dual vision originates from the fact that evolutionary ecologists are more concerned with behavioural decisions and their fitness consequences, while physiologists are by definition more interested in the proximate mechanisms underlying a certain phenotype, such as gonadal growth and ovulation. Experimental studies combining ecological and physiological approaches to the timing of reproduction have increased understanding of this life history trait (e.g. Wingfield 1984; Caro et al. 2006; Caro et al. 2009; Visser et al. 2011a; Schaper et al. 2012) and are thus especially valuable.

In temperate zone birds, the actual process of egg laying is preceded by a physiological cascade mediated by neuroendocrine responses to environmental cues. Egg laying is preceded by the re-activation of the hypothalamic pituitary-gonadal axis by short photoperiods during fall causing the dissipation of photorefractoriness and increased GnRH-1 gene expression (Stevenson & Ball 2009). During winter and early spring the increase in day length stimulates increased secretion of GnRH-I, leading to a release of luteinizing and follicle stimulating hormone (LH and FSH) from the pituitary and a period of gonadal development that lasts several weeks. This photoinduced process, culminating in the laying of the first egg, is fine-tuned by supplementary cues, including temperature and possibly other climatic and phenological cues, including the seasonality of prey items (Wingfield & Kenagy 1991; Wingfield et al. 1992; Dawson 2008).

Due to the difficulties in measuring laying dates in captivity in response to a likely cue, manipulative experiments make use of proxies that are presumed to indicate the timing of egg laying, but are also studied for their own sake (e.g. Storey & Nicholls 1982; Jones 1986; Silverin & Viebke 1994; Wingfield et al. 1996; Wingfield et al. 1997; Sharp et al. 1998; Maney et al. 1999; Wingfield et al. 2003; Dawson & Sharp 2010). Ideally for getting independent data points, pairs of birds would be kept in isolated aviaries, in which environmental variables can be individually regulated. However, this is often not feasible and in many manipulative experiments, the shortcut of examining reproductive physiology instead of a laying date allows for a larger sample size, e.g. many animals (of only one sex) per room or cage, as well as for shorter and less complex experimental designs, as the laying stage does not have to be reached. The most widely used proxies in avian research are, on one hand, gonadal growth, which means the increase in volume of the male left testis, or, more rarely (Ball & Ketterson 2008) the development of the largest follicle in the female ovary, as well as plasma concentrations of gonadotropins, prolactin, or sex steroids, measured either in the blood or in faeces. These measures can be taken at regular intervals during different reproductive stages. More recently, also processes higher upstream in the hypothalamo-pituitary-gonadal (HPG) axis have been added to the physiologist's toolbox, including the release of GnRH-I (Moore et al. 2006; Stevenson & Ball 2009), or even gene expression (Meddle & Follett 1997; Perfito et al. 2010). Emphasis has been placed on photic cues, which determine a broad window for egg laying (Dawson et al. 2001; Sharp 2005), while the influence of supplementary cues has been largely neglected. Conversely, interest in processes closely associated with late reproductive stages, such as the exponential growth phase of the follicle, is increasing, using yolk precursors such as vitellogenin or very low density lipoproteins as proxies (Challenger et al. 2001; Salvante & Williams 2002). This avenue also investigates supplementary cues that might be taken into account in the last days before the actual egg laying takes place.

In studies concentrating on the regulation of the reproductive development by its own means, observations should be made in the context of their adaptive value, most importantly relating to the optimal timing of laying. The way in which an individual female responds to environmental cues affects selection pressures acting on both reproductive physiology, as well as timing of laying (Visser et al. 2010a). Evolution therefore optimizes both the systems of physiological regulation themselves, as well as the behavioural traits that they precede. For example, birds presumably regress their gonads outside the breeding season, because flying with heavier body weights year-round is costly and thus selected against. This makes a phase of gonadal growth in early spring necessary. Also, even though early laying is generally advantageous, as it results in more surviving offspring in that particular year, advancing the physiological development early in spring when food availability is low may impede fitness costs that counterweight these advantages.

The responsiveness to cues might change over developmental stages. It is convenient to assume that a cue, like temperature, which advances the underlying hormonal and gonadal development would also advance egg laying. Indeed, it has often been postulated that temperature influences the timing of reproduction because of an effect on the gonadal development (Wingfield et al. 2003; Dawson 2005a). However, Schaper et al. (2012) showed that in climate-controlled aviaries, moderate spring temperature patterns influenced laying dates of great tits without affecting the timing of gonadal growth or increase in LH concentration.

The assumption that an early rise in gonadotropins would directly translate to early gonadal development, which again would lead to an early onset of laying, has, to our knowledge, never been explicitly tested under controlled conditions. This is basically due to the fact that few experimental studies that report laying dates also measure reproductive physiology, and studies that evaluate reproductive development seldom keep pairs of birds to obtain independent laying dates. In addition, individual variation in physiological measurements is seldom explored in detail, as physiologists mostly report mean values per treatment group in response to environmental stimuli (Williams 2008).

The aim of this study was to use breeding pairs of great tits (*Parus major*) to investigate if the relationship between the timing of individual reproductive development and egg laying is as tight as assumed, or alternatively regulated by different processes, resulting in substantial variation in the interval between, for instance, full gonadal development and laying date. Although the prime objective of the experiments presented here was to show the influence of temperature cues on avian physiology and the onset of laying, the setup allows us to relate the timing of the individual rise in LH, PRL, as well as the growth of testes and ovarian follicles to laying date.

If predictive supplementary cues affect reproductive physiology and consequently egg laying via reproductive development in early spring, we expect a relationship between the timing of a rise in LH, gonadal development and laying date. In contrast, if physiological processes are fine-tuned by different cues, we expect only a loose relationship between these reproductive components and the timing of laying. Additionally, even though a rise in PRL is not causally involved in gonadal growth or the onset of laying, but rather associated with post-laying behaviour, we tested if there would be a correlation between high plasma PRL concentrations pre-laying and laying dates (2011).

Materials and Methods

Birds

This study used 144 first-year breeding pairs of great tits spread over four years. Birds were offspring of known wild parents at the Hoge Veluwe National Park (the Netherlands) and were taken to captivity as complete broods in 2006 to 2009, respectively. On day 10 post-hatching, chicks were taken to the Netherlands Institute of Ecology (Heteren) for hand-raising (Drent et al. 2003).

After independence, fledglings were transferred to single-sex groups in open outdoor aviaries (2 x 4 x 2.5 m), where they were housed until December. Breeding pairs were formed randomly, avoiding sib-matings. Due to fatalities in the young birds, we formed some pairs by using 29 additional spare birds over 4 years, which were hand-raised in the same fashion. On the 1st of December the pairs were placed in climate-controlled aviaries to breed in the next year.

Aviaries

Breeding pairs were housed in 36 separate indoor aviaries (2 x 2 x 2.25 m) under a light regime mimicking the natural photoperiod, which was adapted twice weekly (i.e. for 52°N increasing from 7.45L(light):16.15D(dark) at the winter solstice to 16.30L:7.30D at the summer solstice). Light sources were three high frequency fluorescent light tubes, complemented with an 8 W bulb providing an additional half hour of dawn and dusk. A shaft from the roof (SolaTube), whose opening was synchronized with the light schedule, allowed for supplementary daylight.

The birds were fed *ad libitum* with a constant daily amount of food, consisting of a mixture of minced beef, proteins and vitamin and mineral supplements (Nekton S and Nekton Bio, NEKTON GmbH, Pforzheim), completed by sunflower seeds, fat balls, a mix of dried insects (Carnizoo, Kiezebrink International, Putten), calcium and water for drinking and bathing. Nesting material was provided from March onwards. Birds could choose between two nest boxes, which were inspected for eggs from outside the aviary without disturbance.

Temperature treatments

Over four experimental years, birds were exposed to varying temperature regimes. Each season, a different experimental setup of four temperature treatments was used, each treatment being replicated in a regular design. For a rationale and thorough description of temperature treatments, see Visser et al. (2011a) and Schaper et al. (2012).

In 2007, the 36 pairs of great tits were divided into two groups that differed in the ambient temperature to which they were exposed, with the high temperature treatment set to be always 4°C higher than the cold temperature. From 1st December to the end of February temperatures were kept constant at 4 and 8°C, respectively, after which temperatures gradually increased by 0.65°C per week up to 1st July, reaching 15 and 19°C, respectively. Due to technical difficulties, the 4°C temperature difference was not realized over the entire spring. For example, during the laying period in April/May the overall temperature difference was only 2.5°C (2011).

In 2008, all pairs were exposed to a constant temperature of 15°C from December onwards until summer. In three groups, this temperature was lowered to 7°C in either February, March or April for a month, before it was increased to 15°C again, except for the latest cold period (April), which was maintained until the female initiated laying under cold conditions.

In 2009, there was no seasonal temperature pattern, but a temperature change over the day. Each treatment was composed of a high or low mean with either a high or low day-night amplitude. The two warm treatments were fluctuating around a mean of 14°C (11-17°C, high amplitude, or 13-15°C, low amplitude), while the two cold treatments were fluctuating around 8°C (5-11°C, high amplitude, or 7-9°C, low amplitude) In all cases the lowest daily temperature was reached at 3 am.

In 2010, the setup of the experiment combined two consecutive temperature rises, one during early gonadal development, the other shortly before breeding. All birds were kept at 6°C from December until February. On 8th February, the first two groups experienced a rapid increase in temperature from 6 to 16°C over a course of two weeks, then stayed at 16°C for three or five weeks. On 15th or 29th March, respectively, temperature was increased to 20°C and stayed high during egg laying and moult. Starting on 22nd February, the other two groups were exposed to a more gradual increase in temperature from 6 to 11°C over a course of two weeks, thus experiencing a lower increase rate. These groups then stayed at 11°C for one or three weeks. On 15th or 29th March, respectively, temperatures increased to 15°C for egg laying and moult. Superimposed on the temperature profiles was a day-night rhythm of ± 1°C.

As shown in Visser et al. (2011a) and Schaper et al. (2012), temperature treatments affected neither the increase in plasma luteinizing hormone, nor the development of female or male gonads, while it affected the onset of laying in 2008 and 2010, but not in 2007 and 2009.

Data collection

A blood sample of 100 µl was taken monthly from the jugular vein. Samples were kept on ice until centrifugation, plasma was separated from red blood cells and stored at -80°C. In 2007, blood samples were analyzed for prolactin (PRL), in 2008-2010 for luteinizing hormone (LH). Plasma LH concentrations were determined using a chicken LH radioimmunoassay (Sharp et al. 1987) validated for use in blue tits (Caro et al. 2006). Plasma PRL concentrations were determined using a recombinant derived starling prolactin radioimmunoassay (Bentley et al. 1997). The reaction volume was 60 µl comprising 20 µl of plasma sample or standard, 20 µl of primary antibody (rabbit anti-chicken LH or PRL) and 20 µl of ^{125}I-labeled LH or PRL. The primary antibody was precipitated to separate free and bound ^{125}I label using 20 µl of donkey anti-rabbit precipitating serum and 20 µl of non-immune rabbit serum. All samples from each year were measured in a single assay, in duplicate. The intra-assay coefficient of variation for LH was 6.4% for a high value pool and 8.1% for a low value pool, the minimum detectable dose 0.15 ng/ml. The intra-assay coefficient of variation for the prolactin assay was 6.5% and the minimum detectable dose 1.6 ng/ml.

Alternating in two-week intervals with the blood sampling, a laparotomy was performed monthly to measure gonadal development in 2008-2010. Males were laparotomized from January to July and females up to April in order not to interfere with the laying process. However, in 2009 females were not laparotomized in April, with no apparent effect on the onset of laying, and in 2010 both sexes were not laparotomized in January, as previous years showed little variation in gonad sizes during winter. Birds were unilaterally laparotomized under anaesthesia with isoflurane (Forene, Abbott, Hoofddorp, The Netherlands). Left testis dimensions and diameter of the largest developing follicle in the ovary were measured to the nearest 0.1 mm, using a scale engraved in the ocular of a binocular microscope. Testis volume was calculated as: $V=4/3\ \pi a^2 b$, where a is ½ width and b is ½ length, follicle volume as: $V=4/3\ \pi a^3$, where a is 1/2 width. In April 2008, three females with complete nests were not laparotomized in order not to interfere with the laying decision. Assuming a maximum follicle size of 7 mm^3 for them and including them in the dataset did not qualitatively change the results. Data are not available for all individuals each month due to sampling or assay failure. In total, 17 measures of male and female gonads each and 34 LH values are missing from the dataset.

After nest building was observed, nest boxes were checked daily for eggs. The day that the first egg was found is referred to as the laying date or date of onset of reproduction.

Statistics

The influence of LH concentrations in 2008-2010 on gonadal sizes were analyzed with mixed models (procedure lmer, package lme4 in R 2.10.0, R Development Core Team 2009). Data on gonadal maturation and LH concentrations were natural log-transformed and analyzed per month from February to April. Family was fitted as a random effect and LH of the two previous months as fixed effects. Models also included year as a factor and a variable indicating if a pair was laying afterwards or not. To correct for body size differences in gonadal sizes, tarsus length was included. In 2010, no LH sample was taken in January and early February, hence the effect of LH on gonadal development in February/March was tested in only two years. An alternative analysis in March including all years, so only LH concentrations in March, did not show significant correlations (data not presented). As LH in January was correlated with follicle growth in February, it was additionally included in the model for March. Follicles were not measured in April 2009, restricting this analysis to two years.

The influences of LH concentrations and gonadal sizes in March and April on the timing of egg laying in 2008-2010 were analyzed in a mixed model, including year, as well as female family as a random effect. The influence of PRL concentrations in March and April 2007 on the timing of laying were analyzed in a mixed model, including female family as a random effect.

We used a stepwise model reduction procedure to eliminate non-significant effects. If more than one fixed factor remained significant, mostly in combination with year, the interaction between the variables was additionally tested in the final model. However, none of these interactions were significant (all $P>0.1$, data not shown). We used Markov Chain Monte Carlo sampling to calculate P-values (function pvals.fnc from package languageR, in R 2.10.0). The results are presented including Bayesian 95% highest posterior density credible intervals, equivalent to 95% confidence intervals. As year is given as a multi-level fixed factor in some analyses, a P-value is created for every level in comparison to the year 2008.

Results

Relationship between individual variation in LH titers and gonadal development

Plasma LH concentrations of females and males increased over spring and peaked around March/April (Fig. 5.1). Individuals varied consistently in hormone concentrations over spring and showed substantial variation in the timing of the seasonal increase, leading to substantial differences in LH concentrations in April (Fig. 5.1, unlogged range females: 0.37 to 5.89 ng/ml, males: 0.36 to 5.88 ng/ml). Especially in 2008 and 2009, few individuals either increased earlier than average, or showed elevated titers in general (Fig. 5.1).

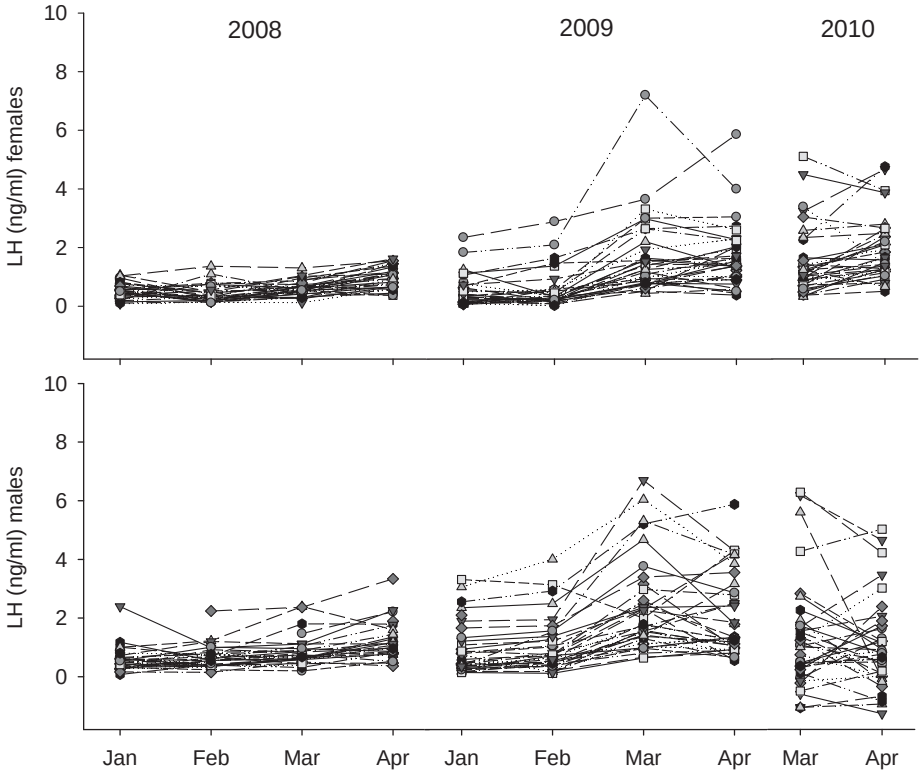

Figure 5.1: Individual patterns of plasma luteinizing hormone (LH) for male and female great tits kept in climate controlled aviaries in 2008-2010. Each line represents an individual bird that was sampled once monthly from January until April in 2008 and in 2009 and from March until April in 2010. Each year comprises of 36 pairs, leading to a sample size of 108 birds.

The development of the largest follicle in the ovary, which is incorporated into the first egg laid, followed an exponential growth pattern, with a slow maturation phase during January to March, well in advance of laying, and an exponential growth phase in April (Fig. 5.2). There were large individual differences in follicle volume in April (Fig. 5.2, unlogged range: 0.03 to 6.37 mm^3), probably caused by variation in the timing of the onset of exponential growth, but females also differed noticeably in early gonadal development.

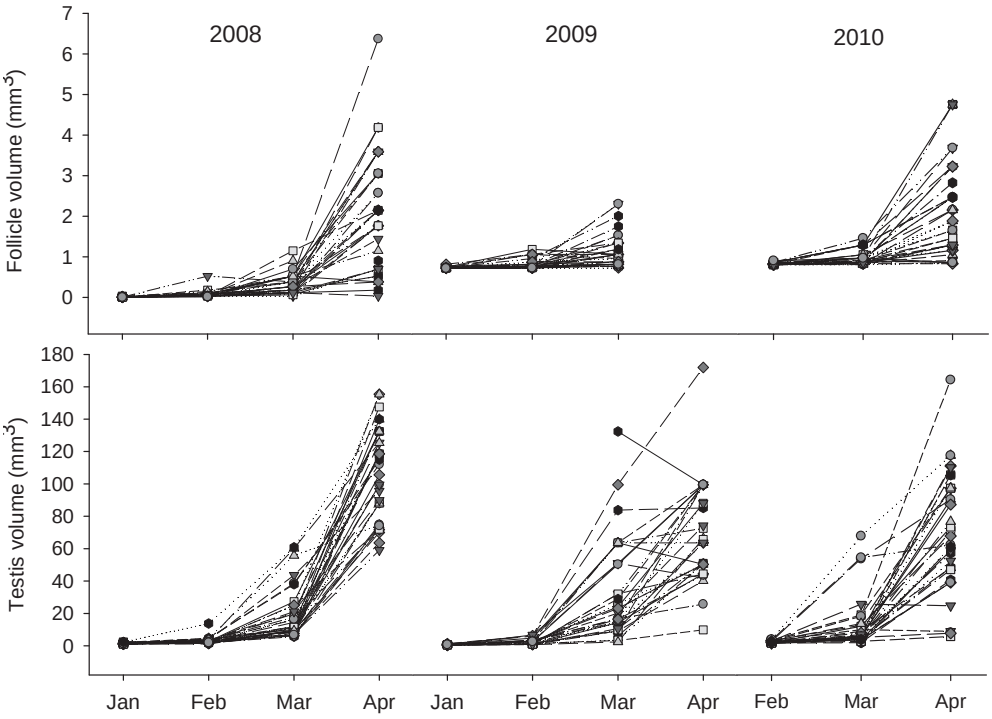

Figure 5.2: Individual gonadal growth patterns for male and female great tits kept in climate controlled aviaries in 2008-2010. Each line represents an individual that was sampled once monthly from January until April in 2008, from January until March in 2009 and from February until April in 2010. Each year comprises of 36 pairs, leading to a sample size of 108 birds.

To investigate the causes of these individual differences, we first explored whether gonadal development was linked to plasma LH concentrations in the same or previous months. Females with high LH concentrations in January had larger follicles in February and March (Table 5.1, Fig. 5.3A,B). Increased LH concentrations in February, March and April did, however, not relate to large follicle sizes in the same or the following month. Non-laying females were characterized by smaller follicles in April compared to females that were going to lay (Table 5.1).

Table 5.1: Relationship between plasma luteinizing hormone (LH) concentrations and gonad sizes in 2008 to 2010. Data on gonadal maturation and LH concentrations were natural log-transformed and analyzed in mixed models per month with family as a random effect. The results are presented including lower and upper Bayesian 95% highest posterior density credible intervals (L 95% HPD, U 95% HPD). As year is given as a multi-level fixed factor in some analyses, a P-value is created for every level compared to 2008. Sample size is given in brackets. Note that birds with missing data were excluded from the analysis.

Response variable	Explanatory variable	Estimate	L 95% HPD	U 95% HPD	t	df	P
Follicles February (log)	LH January (log)	0.46	0.22	0.80	3.39	1	**0.001**
(n=66)	LH February (log)	-0.06	-0.36	0.32	-0.42	1	0.68
	Lay or not	0.34	-0.26	0.89	1.24	1	0.22
	Tarsus	-0.03	-0.07	0.01	-1.59	1	0.12
	Year	0.07	-0.44	0.74	0.21	1	0.83
Follicles March (log)	LH January (log)	0.32	0.09	0.62	2.45	1	**0.017**
(n=103)	LH February (log)	0.10	-0.21	0.42	0.65	1	0.52
	LH March (log)	-0.18	-0.76	0.39	-0.64	1	0.53
	Lay or not	0.35	-0.11	0.91	1.40	1	0.17
	Tarsus	0.02	-0.02	0.05	0.91	1	0.37
	Year	0.26	-0.15	0.81	0.99	1	0.33
Follicles April (log)	LH March (log)	-0.21	-0.83	0.39	-0.70	1	0.49
(n=67)	LH April (log)	0.55	-0.03	1.13	1.90	1	0.062
	Lay or not	0.90	0.36	1.50	3.18	1	**0.002**
	Tarsus	0.01	-0.05	0.07	0.43	1	0.67
	Year	-0.47	-1.04	0.11	-1.71	1	0.092
Testes February (log)	LH January (log)	0.17	-0.02	0.36	1.86	1	0.07
(n=103)	LH February (log)	-0.06	-0.38	0.27	-0.36	1	0.72
	Lay or not	-0.14	-0.44	0.21	-0.91	1	0.37
	Tarsus	<0.01	-0.03	0.04	-0.10	1	0.84
	Year	-0.42	-0.67	-0.14	-2.72	1	**0.008**
Testes March (log)	LH January (log)	-0.29	-0.71	0.11	-1.45	1	0.15
(n=102)	LH February (log)	0.14	-0.12	0.43	1.06	1	0.29
	LH March (log)	0.08	-0.57	0.75	0.26	1	0.80
	Lay or not	0.12	-0.36	0.61	0.48	1	0.63
	Tarsus	0.02	-0.03	0.05	0.91	1	0.37
	Year	0.26	-0.17	0.71	1.22	1	0.23
Testes April (log)	LH March (log)	-0.13	-0.39	0.10	-1.05	1	0.30
(n=100)	LH April (log)	-0.30	-0.51	-0.09	-2.74	1	**0.007**
	Lay or not	0.07	-0.22	0.34	0.52	1	0.60
	Tarsus	0.02	-0.01	0.04	1.41	1	0.16
	Year 2009	-0.34	-0.64	-0.02	-2.09	1	**0.039**
	Year 2010	-0.46	-0.80	-0.14	-2.70	1	**0.008**

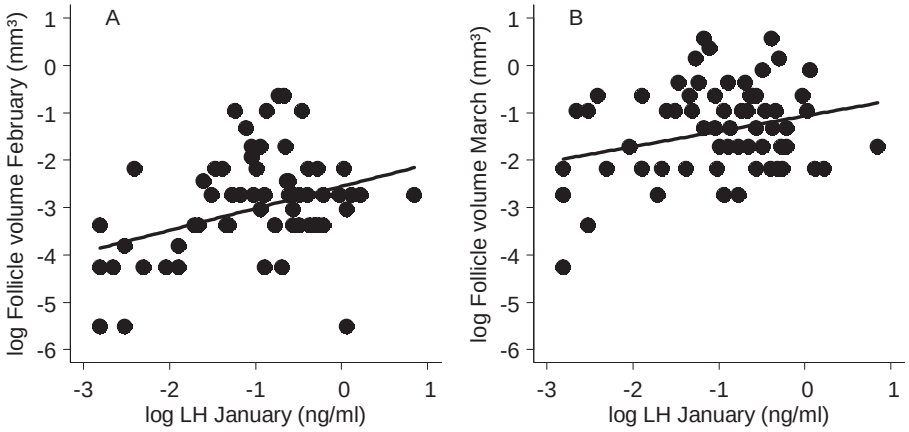

Figure 5.3: Relationship between LH levels in January and follicle development in February and March. Concentrations of plasma LH in females in relation to the volume of the largest developing follicle in the ovary in February (A) and March (B) are given. Data were natural log transformed.

Testes increased exponentially in volume from January/February onwards, in most cases reaching a fully developed state around April (Fig. 5.2), before regressing again in May (data not shown). Individual males varied in both the timing and speed of testis growth, leading to large differences in testis volume in April (Fig. 5.2, unlogged range: 4.54 to 171.91 mm^3). There was no relationship between plasma LH concentrations and testis volume in February to March (Table 5.1). In April, males with larger, fully developed testes had lower circulating LH concentrations than males with still growing testes (Table 5.1, Fig. 5.4). Testes in April were on average further developed in 2008 than 2009 or 2010 (Table 5.1, Fig. 5.2, 5.4). Testis volume did not differ between males paired to females that were going to lay eggs or not (Table 5.1).

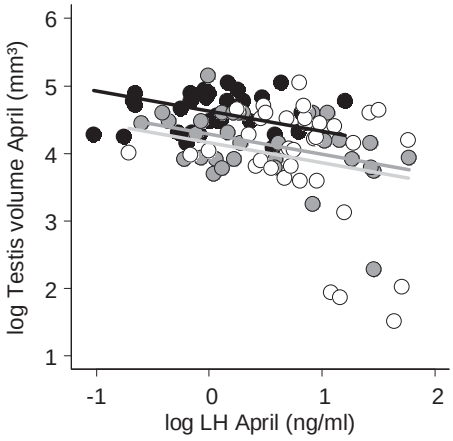

Figure 5.4: Relationship between LH levels in April and testis development in April. Concentrations of plasma LH in males in relation to the volume of the left testis in April in the years 2008 (closed circles), 2009 (grey circles) and 2010 (open circles) are given. Data were natural log transformed.

Relationship between individual variation in PRL titers and the onset of laying

In 2007, plasma PRL concentrations increased over spring (Fig. 5.5), whereas peak concentrations were reached in May (data not shown). Similar to LH, there was individual variation in the timing and speed of increase in early spring PRL titers, leading to substantial differences in PRL concentrations in April (Fig. 5.5, unlogged range females: 6.49 to 70.73 ng/ml, males: 1.82 to 103.77 ng/ml). There was no relationship between PRL levels in March or April and the onset of laying (all $p>0.1$, data not shown).

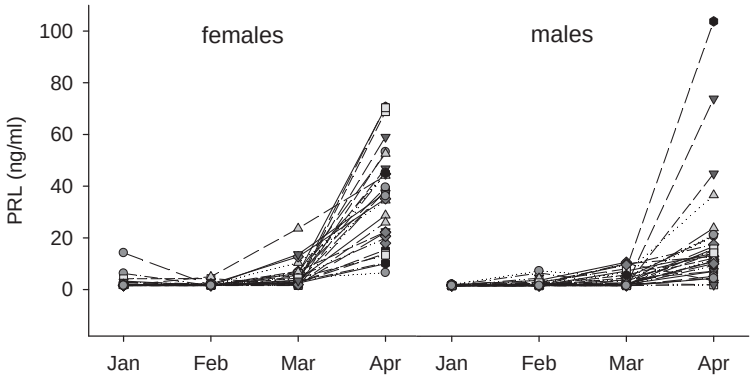

Figure 5.5: Individual patterns of plasma prolactin (PRL) for male and female great tits kept in climate controlled aviaries in 2007. Each line represents an individual bird that was sampled once monthly from January until April in 2007. The data consists of 36 pairs.

Relationship between LH titers, gonadal development and the onset of laying

Egg laying started in mid-April, but was on average later in 2008 (Table 5.2, Fig. 5.6), when the variation in laying dates between females was also largest. The onset of laying was not related to plasma LH concentrations in previous months (Table 5.2). Neither the size of the largest developing follicle in April, nor the development of the partner's testis in April predicted laying date (Table 5.2). However, females with large follicles in March, quite in advance of the rapid growth phase, laid on average earlier than females with less developed follicles in March (Table 5.2, Fig. 5.6A). In addition, males with larger testes in March had mates that initiated laying early (Table 5.2, Fig. 5.6B). This was true even though females with further developed follicles in March were not paired to males with larger testes (linear model, t=0.33, P=0.7). Yet, especially in 2008, the relationship between gonad size in March and laying dates was not particularly tight (Fig. 5.6). In a linear model only including follicle volume or testis volume by themselves, gonadal size in March only explained a small amount of the variation in laying dates, 1.4% in case of testes and 2.3% in case of follicles, showing that male and female gonad sizes cannot be indicative of the timing of the laying event.

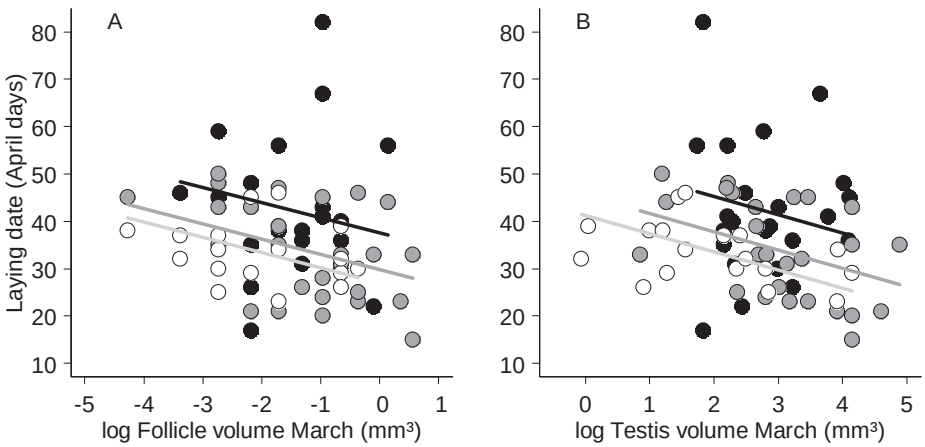

Figure 5.6: Relationship between gonad sizes and laying dates in 2008-2010. Volume of the largest developing follicle in the ovary (A) and volume of the left testis (B) in relation to the pair's laying date in the years 2008 (closed circles), 2009 (grey circles) and 2010 (open circles) are given. Gonad data were natural log transformed. Laying dates are given in April days, where 1=1st April.

Table 5.2: Relationship between plasma luteinizing hormone (LH) concentrations, gonad sizes and laying dates in 2008 to 2010. Data on gonadal maturation and LH concentrations were natural log-transformed and analyzed in a mixed model with female family as a random effect. The results are presented including lower and upper Bayesian 95% highest posterior density credible intervals (L 95% HPD, U 95% HPD). As year is given as a multi-level fixed factor, a P-value is created for every level compared to 2008.

Response variable	Explanatory variable	Estimate	L 95% HPD	U 95% HPD	t	df	P
Laying date	Follicle volume March (log)	-3.20	-5.76	-0.65	-2.50	1	**0.02**
(n=65)	Follicle volume April (log)	0.01	-7.32	6.86	<0.01	1	1
	Testis volume March (log)	-3.84	-6.55	-1.07	-2.77	1	**0.008**
	Testis volume April (log)	-4.37	-9.97	1.87	-1.49	1	0.14
	LH female March (log)	2.34	-2.59	7.71	0.91	1	0.37
	LH female April (log)	-2.84	-9.99	4.07	-0.81	1	0.42
	LH male March (log)	2.45	-3.47	8.33	0.82	1	0.42
	LH male April (log)	-3.87	-8.88	1.00	-1.55	1	0.13
	Year 2009	-6.84	-12.97	-0.22	-2.20	1	**0.032**
	Year 2010	-13.62	-20.76	-6.25	-3.70	1	**0.001**

Discussion

We kept pairs of great tits under controlled conditions to investigate if pre-laying endocrine changes and gonadal growth correlated with each other and whether their timing was related to the onset of laying. These physiological measurements, often used as proxies for breeding phenology, showed consistent individual variation, but were at best weakly correlated to each other or to the onset of laying. In consequence, laying dates could not be predicted by comparing sizes of the largest follicles in late spring.

Females adjusted their timing of laying independent of gonad development

While some females had already functional gonads in April, they seemed to postpone laying in response to environmental information. Visser et al. (2011a) and Schaper et al. (2012) demonstrated that while in this set of birds the pre-laying physiological development was not influenced by temperature treatments, females adjusted their laying date when the right temperature cues were provided. The disconnection between individual hormone levels, gonad sizes and laying dates presented here further validates that reproductive development is not the factor that constrains laying. The ability to fine-tune the onset of laying to environmental conditions irrespective of large differences in developmental state emphasizes the importance of supplementary cues close to laying. Variation in testis size of their mates predicted female laying dates equally little, which is less surprising, as a laying date is primarily a female-driven trait (Caro et al. 2009). In comparison, in one of the few field experiments measuring endocrinology and reproductive physiology in combination

with laying dates, Caro et al. (2006) showed that two blue tit (*Cyanistes caeruleus*) populations breeding one month apart only showed a two-week asynchrony in the seasonal patterns of plasma LH and testosterone and a comparably small difference in the timing of testis growth. Our standardized aviary setup did specifically not provide the complex of correlated cues that are available for birds in nature, e.g. photoperiod, temperature, visual, olfactory and seasonal food cues, which in combination might result in the closer relationship between the timing of endocrine and gonad development and laying date. Our findings, pointing at the disconnection between the timing of gonadal development and laying dates under standardized conditions, have implications for physiological studies traditionally concentrating on male reproductive development to determine the effect of environmental cues on timing of reproduction. Herewith we emphasize once more the importance of measuring laying dates complementary to reproductive physiology to make inferences about seasonal timing of reproduction.

Individuals showed unexplained variation in pre-laying physiology

Plasma hormone concentrations, as well as gonadal development of females and males showed phenotypic variation, even under controlled conditions of *ad libitum* food, natural photoperiod and standardized social cues, e.g. keeping birds in individual pairs. Part of the variation in hormone titers could be due to differences in sampling time of day, which is however unlikely responsible for the consistent individual variation found here. On top of this variation, a linear relationship between plasma hormone concentrations and effector systems can only be assumed within certain limits (Ball & Balthazart 2008). Downstream responses will be modified by individual variation in, for example, the amount of binding globulines or hormone receptors. It would be very worthwhile to further explore causes and mechanisms of the unexplained plasticity in endocrine systems and reproductive physiology, as well as its functional significance, heritability and adaptive value (Ball & Balthazart 2008; Williams 2008).

Variation in gonadal growth was not related to variation in LH titers in most months

Follicle growth was more closely linked to LH concentrations in January than in subsequent months. LH levels are known to rise after photostimulation in spring (Sharp 2005), and in this setup, day length was increased following a seasonal pattern, leading to a very natural increase in LH concentrations. At the time of measurements in January, birds were exposed to ca. 9:15 h of light, including dawn and dusk, likely not enough to fully photostimulate the birds. However, there was remarkable variation in LH levels at this point. We can only speculate that these were (genetic) differences in sensitivity to the seasonal increase in day length, which subsequently affected initial gonadal growth rates. The relationship between increasing LH levels and gonadal growth was less tight in the following months, which could mean that gonadal growth

was fine-tuned by other internal or external cues not integrated via the LH pathway. Currently it is impossible to measure avian follicle stimulating hormone (FSH), which could likely form the link between environmental cues and gonadal development. In general, when we assume that cues affect gonadal growth also via different pathways, we expect the relationship between any gonadotropin and gonadal development to be less tight in the late stages of gonadal development when supplementary cues become more influential.

The negative relationship between LH levels and testis size in males in April exemplifies the difficulty to draw conclusions from punctual or stochastic samples. In this case, high levels of LH were related to small testes, presumably because in males with fully-grown testes LH concentrations decreased already before April due to steroid feedback. Caro et al. (2006) found in Corsican blue tits that at the time of laying, when males had fully functional testes, plasma LH levels were similarly decreasing as shown here. Caution is therefore needed when making assumptions about timing of reproduction based on the comparison of punctual physiological measures, which can potentially miss a developmental peak. It also has to be cautioned here that gonad size does not directly indicate functionality, again pointing towards the importance of making behavioural observations to complement physiological measures.

Variation in pre-laying prolactin titers was not related to timing of laying

Prolactin (PRL) concentrations were not elevated in females or males that were closer to laying, in contrast with a recent study by Ouyang et al. (2011). PRL is associated with incubation and parental behaviour, and thus exploring the individual variation in PRL levels close to laying in combination with reproductive performance or the timing of incubation behaviour would be most interesting, but goes beyond the scope of this paper.

Approaches to variation in pre-laying reproductive endocrinology and physiology

It is crucial to investigate, under controlled conditions, the variation in endocrine and physiological mechanisms that cause individual variation in the onset of reproduction. For this, there are three complementary avenues that need to be explored.

Firstly, we need to find out in how far non-photic cues regulate reproductive pathways from an early stage onwards. For example, it has been shown that LH levels in male songbirds, even though primarily regulated by photoperiod, increase in response to environmental stimuli, such as the onset of rain (Small et al. 2008), or the presence of leafing birch branches (Visser et al. 2002, but see Schaper et al. 2011). Furthermore, if LH plasma concentrations are only loosely regulating gonadal development, the question remains which external or internal information is reflected in elevated LH concentrations and which mechanisms might be affected further downstream.

Secondly, we need to concentrate our efforts on pathways unrelated to gonadotropins and gonadal growth that can accommodate the transduction of supplementary cues to fine-tune the onset of laying. The disconnection between relatively late stages of gonadal development and the onset of laying shown here exemplifies the scope for such a mechanism, for example accommodating temperature cues.

Thirdly and finally, we need to identify genetic variation underlying both the way in which environmental information is integrated and transduced into a physiological and behavioural phenotype. The genetic mechanisms maintaining plasticity in the physiological phenotype need to be identified if we ultimately want to predict how fast and to what extend animals can adapt their timing of seasonal breeding to changes in their environment, including climate change.

Summary

Our findings stress that stages of avian reproductive development until egg laying are regulated by different processes and are likely to be responsive to different stimulatory cues. This calls for the investigation of causes of this intriguing individual variation in endocrine systems and reproductive physiology for its own sake. Ultimately, these processes are culminating in egg laying, and acknowledging the paradox of the missing connectivity between reproductive physiology and the laying decision is essential to fully understand effects of environmental variation on timing of reproduction.

Acknowledgements

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Chapter 6

Sonja V. Schaper, Phillip Gienapp, Alistair Dawson
and Marcel E. Visser

Timing of gonad development is heritable in a wild song bird

Submitted manuscript

Abstract

Many organisms advance their seasonal reproduction in response to global warming. In vertebrates, these shifts are ultimately constrained by the time required for photoperiodically-controlled gonadal development. As there is no phenotypic plasticity in this developmental rate to temperature, heritable variation in reproductive physiology is a crucial prerequisite for adaptation. We show that the extent of ovarian follicle development in early spring is highly heritable in a wild bird, the great tit (*Parus major*), in which we observe directional selection for advanced egg laying. This genetic control decreased as spring advanced, demonstrating a progressive modifying effect of non-genetic factors. Our results clearly show that there is heritable variation in the physiological mechanism underlying a female life history trait, allowing genetic adaptation to climate change.

Introduction

The natural world is changing at an unprecedented rate in response to climate change (Walther et al. 2002; Parmesan & Yohe 2003; Root et al. 2003), and global warming has led many organisms to reproduce earlier in the season (Beebee 1995; Crick et al. 1997a; Forchhammer et al. 1998; Visser et al. 1998; Brown et al. 1999; Charmantier et al. 2008). Small songbirds, which aim to time egg-laying such that the time of maximum nestling growth coincides with maximum food availability in the environment (Rowan 1926; Lack 1968), currently face the problem of an increasing mismatch of their breeding season with the short period of high food abundance required to feed their young (Visser et al. 1998). This phenological mismatch results in a disruption of population dynamics with wider implications for ecosystem functioning (Both et al. 2006; Møller et al. 2008; Jones & Cresswell 2010).

Time of egg-laying is affected by the rate of development of the underlying physiological mechanisms, as egg laying can only occur following full gonadal maturation in both sexes, and this process can take several months, typically starting during winter (e.g. Dawson 2003; Dawson 2005a; Visser et al. 2011a; Schaper et al. 2012). Avian testes increase in size several hundred fold during this period (Dawson et al. 2001). Birds use the annual cycle in photoperiod as a predictive cue to time gonadal maturation (Dawson et al. 2001), which commences following the dissipation of photorefractoriness by short photoperiods during late autumn or winter. Thereafter, the rate of maturation, at least during the earlier stages, is proportional to photoperiod (Farner & Wilson 1957; Farner et al. 1966) and thus increases as photoperiod increases during spring, culminating in full maturation in time for laying. In later developmental stages, other environmental cues may be used to determine the exact time of laying (Wingfield & Kenagy 1991; Wingfield et al. 1992; Dawson 2008; Schaper et al. 2012).

Climate warming will lead to an advance in the availability of food resources necessary to feed young since the rate of invertebrate development is temperature dependent (van Asch et al. 2007). Although there is evidence that the time of first egg-laying is affected by ambient spring temperatures (Visser et al. 2009; Schaper et al. 2012) and that it has advanced in many species during recent years (Crick et al. 1997a; Winkel & Hudde 1997; McCleery & Perrins 1998), this is often insufficient to compensate for the advance in food resources, and so a mismatch in timing occurs (Visser et al. 1998). Earlier laying in warmer springs may suggest that temperature has an effect on the rate of photoperiodically-induced gonadal maturation. However, this is not the case; in studies on starlings (Dawson 2005a) and great tits (Visser et al. 2011a; Schaper et al. 2012) exposed to a simulated natural increase in photoperiod during spring, ambient temperature had no effect on the timing or rate of gonadal maturation. The observed temperature-related plasticity of egg-laying has hence to be due to physiological processes or behavioural decisions taking place after full gonadal maturation. The fixed response of reproductive physiology to photoperiod will therefore ultimately constrain the ability of birds to advance laying dates to compensate for the earlier appearance in food resources.

Adaptation to climate change can only occur if there is heritable variation in physiological responses to photoperiod. Quantitative genetic analyses have shown that the precise time that the first egg is laid is phenotypically plastic and fine-tuned in response to increasing spring temperatures, that individual females differ in their plasticity and that this variation is heritable (Husby et al. 2011, but see Brommer & Rattiste 2008; Husby et al. 2010). However, an analysis of the variation, plasticity and heritability of the underlying reproductive physiology is currently lacking. Even when behavioural decisions are plastic in response to environmental temperature changes, responses to global warming will be, at some point, constrained by the timing of the mechanisms controlling the underlying reproductive physiology.

In this study, we aimed to measure variation and heritability in the rate of testicular and ovarian maturation in great tits in response to simulated natural changes in photoperiod. Between 2007 and 2010, four separate experiments were carried out under controlled conditions to investigate the effects of different temperature regimes on the timing and rate of gonadal maturation. In each of these experiments, pairs of birds were kept in climate-controlled aviaries under a simulated natural increase in photoperiod and exposed to different temperature regimes. By applying a between-sibling comparison we demonstrate heritable variability in the extent of follicle growth in early spring in this small songbird.

Materials and Methods

Birds

In total, we used 144 one-year old great tit pairs in these experiments over four years. The birds were the offspring of 40 wild pairs from a long-term studied population that we chose for having either early or late laying dates (see scheme in Fig. 6.1). Paternity by the social father was verified (Saladin et al. 2003). We hand-raised the chicks from day 10 post-hatching onwards and after nutritional independence kept them in single-sex groups in outdoor aviaries. We could thus assess full-sibling family resemblance by measuring reproductive timing in sisters and brothers raised and kept under standardized conditions, after being exposed to the same early rearing environment within the family. In December, we formed non-sibling pairs within a pool of five early or five late laying families per year. The parents' laying dates did not affect the offspring's gonadal development (Schaper et al. 2012).

Housing conditions

Breeding pairs were housed in 36 separate climate-controlled aviaries (2 x 2 x 2.25 m). They received a light regime mimicking the natural photoperiod, with step changes twice weekly. Light sources were three high frequency fluorescent light tubes, complemented with a 8 W bulb providing an additional half hour of light at dawn and dusk. A shaft from the roof, whose opening was synchronized with the light schedule, allowed for supplementary daylight. The birds were fed *ad libitum* with a constant daily amount of food (Visser et al. 2011a; Schaper et al. 2012) and water for drinking and bathing. We provided nesting material from March onwards.

We exposed the breeding pairs to experimental temperature treatments which varied over years, but did not affect gonadal development (for details and rationale see Visser et al. 2011a; Schaper et al. 2012).

In 2007, we divided the 36 pairs into two groups differing in the ambient temperature to which they were exposed, with the cold temperature treatment 4°C lower than the warm temperature. From December to March temperatures were kept constant at 4 and 8°C, respectively, after which we gradually increased temperatures by 0.65°C per week until July, reaching 15 and 19°C, respectively. Due to technical difficulties, the 4°C temperature difference was not realized over the entire spring. For example, during the laying period in April/May the overall temperature difference was only 2.5°C. In 2008, we divided the pairs into four groups, all of which were exposed to a constant temperature of 15°C from December onwards. In three groups, this temperature was lowered to 7°C in February, March or April for a month, before being increased to 15°C again. In 2009, there was no seasonal temperature pattern, but we changed temperature over the day. Each treatment was composed of a high or low mean with

either a high or low day-night amplitude. The two warm treatments fluctuated around 14°C (11-17°C or 13-15°C), the two cold treatments around 8°C (5-11°C or 7-9°C). In 2010, we kept all birds at 6°C until February. On 8th February, two groups experienced a linear increase in temperature from 6 to 16°C over the course of two weeks and were then kept at 16°C for three or five weeks, after which temperature was increased to 20°C. Starting on 22nd February, we exposed the other two groups to an increase from 6 to 11°C over the course of two weeks and were then kept at 11°C for one or three weeks, after which we increased temperatures to 15°C.

Data collection

We measured the size of the testis or largest ovarian follicle monthly via laparotomy, except in January 2010 and females in April 2009. Birds were unilaterally laparotomized under isoflurane anaesthesia (Forene, Abbott, Hoofddorp, The Netherlands). Left testis dimensions and diameter of the largest follicle in the ovary were measured to the nearest 0.1 mm, using a scale engraved in the ocular of a binocular microscope. We calculated testis volume as: $V=4/3\pi a^2 b$, where a is width/2 and b is length/2, and follicle volume as: $V=4/3\pi a^3$, where a is width/2. We could not sample all birds successfully monthly, leading to varying sample sizes.

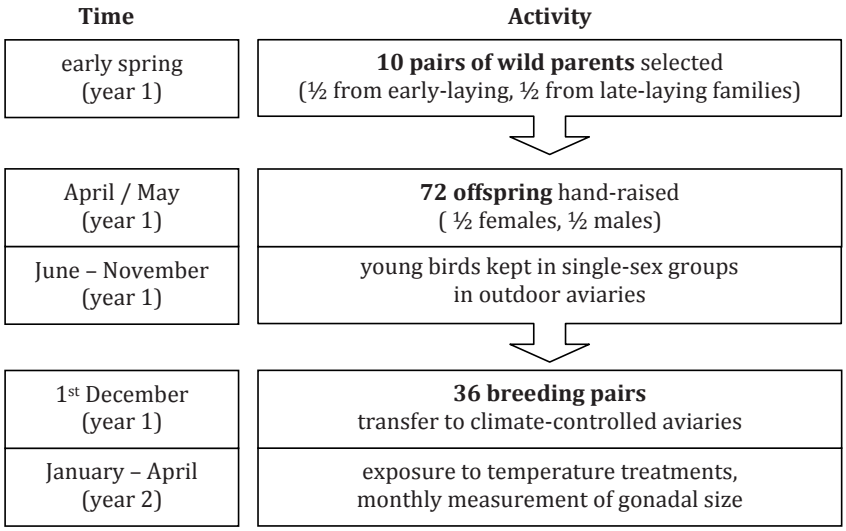

Figure 6.1: Origins and housing conditions of captive birds. Scheme of the origins and history of housing conditions of the experimental breeding pairs in the aviary setup. The setup was repeated over four years from 2007 to 2010, so that gonadal growth from 144 female and 144 male captive great tits from 40 families was measured in total.

Statistical analyses

In calculating heritabilities, we log-transformed gonad volumes and analyzed them separately for each sex and month using a special form of a statistical mixed model, the so-called 'animal model' (Kruuk 2004). The pedigree included up to the grandparental generation, but this was incomplete for some broods. Only families with at least two siblings of the measured sex in a month were included. To test whether additive genetic variance and heritabilities varied among months we chose not to use a random regression animal model, which would test whether individual slopes differ genetically, because the assumption of linear slopes may not be satisfied. We aimed to test the interaction between month and the additive genetic effect within the animal model framework, but these models were too complex. We therefore tested the interaction between month, as a fixed factor, and family, as a random effect, in a mixed model. Since there were few pedigree links between parents of sib-groups, our pedigree structure resembled closely a full-sib breeding design and this approach yields very similar results to an animal model including the complete pedigree, while being computationally less complex. As a variance-covariance matrix was fitted, i.e. correlations of the family-effect among months were not constrained, a likelihood-ratio test with nine degrees of freedom was used. Due to repeated measurements, individual was fitted as a random effect. We included tarsus length as a covariate to correct for body size. By fitting a fixed year effect, we avoided introducing bias due to variation in environmental conditions between birth years, variation in the timing of monthly measurements, or experimental temperatures between years. Models were run with ASReml 3 (VSN International). Significance of narrow-sense heritability (h^2) was tested by comparing models with and without the additive genetic effect fitted using a likelihood-ratio test with one degree of freedom. We hypothesised that the onset of reproductive development differs between the 40 families, indicating a genetic component, and that this would result in gonadal size differences at particular dates.

Results & Discussion

From January to April, the size of the largest ovarian follicle and the size of the testes increased exponentially as photoperiod increased naturally (Fig. 6.2A,B). This photoinduced gonadal maturation was not affected by temperature (Schaper et al. 2012). These data, in combination with pedigree data, were used to calculate monthly heritabilities of the extent of gonadal maturation.

We found clear heritable variation in the maturation of the largest ovarian follicles from the earliest stages of ovarian development onwards (Table 6.1, Fig. 6.3A). This demonstrates that the photoinduced extent of follicle growth is partly genetically controlled. We also show that the genetic control of follicle size, which accounts for more than 70% of the variation in this trait in January, decreases progressively

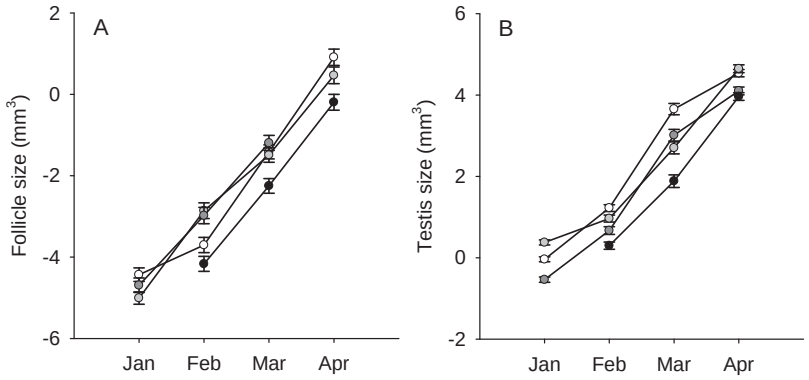

Figure 6.2: Growth of the largest ovarian follicle (A) or left testis (B) before the start of seasonal reproduction in great tits in 2007-2010. Females and males were measured from January to April in 2007 (open circles), 2008 (light grey circles), 2009 (dark grey circles) and 2010 (closed circles). Gonad volume was not measured in January 2010 and in females in April 2009. Data are log-transformed. Means (± 1 SE) are given.

	Follicle volume (log) females				Testis volume (log) males			
month	January	February	March	April	January	February	March	April
V_P	0.807 (0.144)	1.118 (0.155)	1.011 (0.135)	1.208 (0.189)	0.116 (0.018)	0.236 (0.032)	0.672 (0.089)	0.280 (0.036)
V_A	0.588 (0.287)	0.577 (0.282)	0.335 (0.229)	0.175 (0.319)	0.015 (0.030)	0.074 (0.056)	0.163 (0.143)	0.021 (0.054)
h^2	0.729 (0.274)	0.516 (0.216)	0.332 (0.210)	0.145 (0.259)	0.131 (0.254)	0.313 (0.222)	0.243 (0.204)	0.076 (0.193)
n (individuals)	85	127	127	89	90	122	126	127
n (families)	27	38	37	25	28	38	39	40
χ^2	7.58	10.56	3.54	0.32	0.27	2.6	1.96	0.17
P	**0.006**	**0.001**	0.06	0.57	0.61	0.11	0.16	0.68

Table 6.1: Results from animal model analyses. Variance components, heritabilities and sample sizes (n) from animal model analyses of logged gonad size, separated by sex and month. V_P is the total phenotypic variance and V_A the additive genetic component. The heritability (h^2) is the proportion of the variance explained by the additive genetic effect (V_A/V_P). Estimates are followed by their standard errors, in brackets. Significances refer to heritabilities.

between February and April (Table 6.1, Fig. 6.3A). This strongly suggests that the shared genetic element does not lie in the speed of gonadal development, because this would lead to higher resemblance between related females at later, rather than earlier, stages. The decreasing heritability (Table 6.1, Fig 6.3A) is probably the result of a steady accumulation of the effects of random variation in growth rate.

In contrast to females, the extent of gonadal development in males was not determined by genes shared by brothers (Table 6.1, Fig. 6.3B). When corrected for body size, this variation in the onset of testis development could be the result of slight differences in body condition.

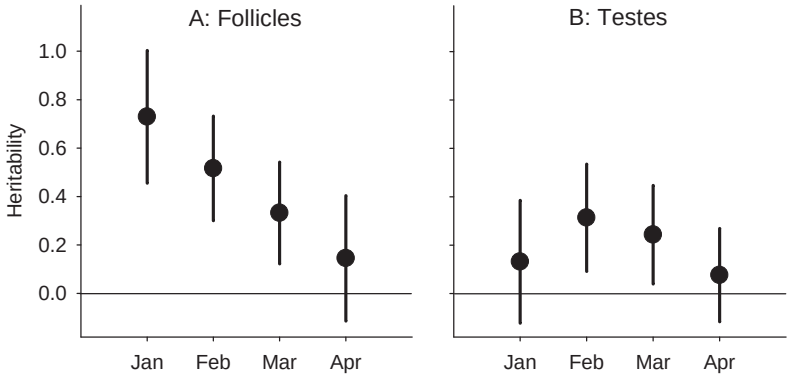

Figure 6.3: Heritabilities of the largest ovarian follicle volume (A) and testis volume (B) before the start of seasonal reproduction in great tits. Heritabilities (± 1 SE) of follicle volume decreased from January to April (χ^2=22.0, df=9, P=0.009) and differed from zero in January (h^2=0.729, df=1, P=0.006) and February (h^2=0.52, df=1, P=0.001), but not any more in March (h^2=0.33, df=1, P=0.06). Heritabilities of testis volume did not differ from zero (all p>0.05).

We thus identified the extent of early gonadal growth in females as a heritable avian reproductive trait. We show, for the first time to our knowledge, that genetic effects on the reproductive system are initially high, but diminish during seasonal development. Due to their heritability, reproductive processes, such as gonadal growth in females, can respond to selection by micro-evolution. Such micro-evolution is needed as recent climate warming currently favours an advance in the onset of laying of about two weeks. This may be achieved by plasticity in the final rapid gonadal maturation phase. However, if the trend to an earlier onset of laying continues, the observed variation in the initial rate of ovarian growth will become more important in accommodating this trend and may eventually limit it. Only genetic shifts in the time of gonadal development can then further a shift in egg laying date.

Our results have implications for understanding genetic variation in key avian life-history traits, which are changing in response to climate warming in different ecosystems worldwide (Forchhammer et al. 1998; Visser et al. 1998; Hughes 2000; Parmesan & Yohe 2003; Barbraud & Weimerskirch 2006). These changes are at least partly based on selection of underlying physiological mechanisms rather than selection of the life-history trait itself. Components of the mechanism can show variation, but may not be plastic or heritable, thereby restricting an adaptive change in the trait value in response to climate change (Visser 2008). Integration of quantitative genetics and developmental physiology, in combination with an ecological understanding of natural selection pressures, is needed to develop predictive models of the responses of bird populations to climate change.

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Chapter 7

Sonja V. Schaper and Marcel E. Visser

Great tits provided with *ad libitum* food lay larger eggs when exposed to colder temperatures

Submitted manuscript

Abstract

The amount of resources deposited into an avian egg is thought to be actively regulated by the female and varies both between and within clutches. Larger egg size enhances offspring traits ranging from hatching success to fledgling survival, but as a trade-off, laying large eggs also infers energetic costs to the female. In income breeders, females usually lay larger eggs later in the season, when temperatures and food availability are higher. Egg size is therefore believed to be affected by the daily amount of energy available to produce an egg under cold conditions, but it is less well known in how far temperature exerts direct effects on egg size. We show that great tits with access to *ad libitum* food breeding in climate-controlled aviaries considerably varied their investments in eggs. Size of an individual egg was best predicted by mean temperatures one week pre-laying, with females laying larger, rather than smaller, eggs under colder conditions. There was no effect of laying sequence, but eggs increased in size over the season. The degree of daily temperature fluctuations did not influence egg size. In addition to a large between-female variation, sisters were more similar to each other than unrelated females, showing that egg size does also reflect heritable intrinsic properties of the female. Natural variation in egg size is thus not only determined by energy-limitation, but is also due to females allocating more resources to eggs laid in colder environments, when larger eggs are probably less prone to chilling and increase early survival of the chicks. That the positive correlation between temperature and egg investments is reversed under *ad libitum* food demonstrates that wild great tits trade-off own condition with survival prospects of their chicks as a function of available food, not ambient temperature.

Introduction

Avian egg size is a trait which has received much attention in recent years, but causes and consequences of individual variation between eggs remain poorly understood. Being iteroparous, avian females in general have to make a decision how much they invest in their offspring relative to their own self-maintenance, which will affect survival and future reproductive events. The first costly investment in the offspring involves the amount of nutrients deposited into the egg (Visser & Lessells 2001), which is mirrored in absolute size of the egg. Egg size (measured as volume or mass) can have profound effects on a range of fitness-related offspring traits. A recent meta-analysis by Krist (2011) based on 283 studies of 162 avian species found a strong correlation between offspring quality traits, assessed from the egg stage to the post-fledging stage, and egg size. Egg size appeared to affect hatching success and egg fertility. Around hatching, it was correlated with hatchling's body mass and to a lesser extent body condition, skeletal size and wing/feather length. The influence of egg size diminished as chicks grew older, affecting mostly morphological traits, but also survival and growth rates, at the nestling stage. Due to the small number of evaluated studies, significant effects in the post-fledging stage were only found for

survival. These strong effects were not diminished when constraining the analysis to studies employing a cross-fostering design, so this effect was probably not due to territory or parental quality, but rather the egg environment shaped by the mother. That egg size mostly affected juvenile survival (hatching success, chick weight and growth trajectories) might also be due to the concentration of studies on this aspect, as compared to adult survival and fecundity. Still, it is obvious that avian egg size is an important factor affecting the fitness of individual chicks. Additionally, as larger eggs hatch larger and faster-growing chicks (Krist 2011), an investment in eggs also reduces the costs for caring for the young post-hatching.

Given the large fitness consequences of egg size, it is striking that there is considerable within-species variation in egg size, with females often laying smaller eggs than would probably be optimal for the fitness of an individual chick, assuming that larger egg size is advantageous in any circumstances. The general belief in this regard is that female birds are able to actively regulate egg size. Due to the energetic demands females face when producing an egg, which in passerines can be about 40-50% of their total daily energy budget (Perrins 1996), there will be a trade-off between the direct investment in eggs and the amount of energy available for self-maintenance and future reproduction, including subsequent clutches laid in the same season. Females should therefore carefully balance the fitness prospects of their chicks with their own condition. This trade-off is likely to result in larger optimal egg size (from the female's perspective) under more favourable conditions. In income breeders, such as small passerines, egg size should hence be directly affected by ambient temperature and food abundance. Both foraging success and thermoregulatory costs of an individual female are related to the seasonal changes in ambient temperature. It is therefore not surprising that in nature, egg size is confounded with date in the season (Perrins 1996), which is highly correlated with both food abundance and temperature.

In a review on causes of avian egg size variation, Christians (2002) found both positive and negative effects of ambient temperatures on egg sizes in correlative field studies, which, however, explained at most 10-15% of the variation. Nager & Zandt (1994) measured egg volumes of first clutches of yearling great tit females in relation to ambient temperature three days pre-laying and food abundance. They found that environmental conditions only explained a small proportion of the observed variation in egg volume, but females laid smaller eggs at lower minimum temperatures, whereas mean temperature had no effect. In this study, young females early in the season were affected more strongly by temperature than older birds. However, temperature only influenced relative egg size when mean temperature during laying was below 10°C (van Noordwijk 1984; Nager & Zandt 1994). For an average-weight yearling female egg volume increased by 0.013 cm^3/°C. In addition, low food abundance only led to small egg sizes under cold temperature conditions. In the only field study that directly manipulated temperatures and investigated the effects on laying dates and egg

sizes, Nager & van Noordwijk (1992) found that great tit females roosting in heated, as opposed to cooled, nestboxes apparently saved energy from thermoregulation, which resulted in laying of larger eggs. They observed an increase in egg volume of 0.08cm^3/°C, which corresponds to a 14% increase in warmer nest boxes. However, ambient temperature during the experimental period was usually below 10°C (heated boxes: 5.6°C, cooled boxes 2.7°C during the night) and the relationship between egg-size and environmental conditions might not be linear (van Noordwijk 1984; Nager & Zandt 1994).

Even though environmental conditions strongly affect the size of each individual egg, birds often show a lack of flexibility in egg size within a clutch, and overall eggs of individual females are less variable than eggs from different females breeding in a similar environment (Christians 2002). Egg size might be constrained by the condition of the individual female and her physical ability to convert energy into the developing egg, which in turn depends on her foraging abilities, as well as social status and territory. Only 30% of egg size-variation is normally found within clutches (Christians 2002). Both female quality and selection for a constant egg size within a clutch could explain this comparably small variability. As egg size seems to affect hatchling size and growth rate (Krist 2011), passerine females should be adapted not to produce broods of chicks with asynchronous growth trajectories, which increases the chance to loose proportionally weaker nestlings, and therefore experience selection pressures to keep egg size constant within a clutch. This selection for an invariable egg size could limit the female's selective advantage of being flexible to adapt maternal investments to the current environment on a daily basis.

To summarize, in birds there is evidently individual variation in egg size between females, between clutches of the same female, but also, to a lesser extent, within the same clutch. An excellent model species to study the causes of the large individual variation in egg size are small passerines, as they lay comparably large clutches. In case of the great tits in our population the clutch size in recent years is about 7-12 eggs (L. te Marvelde, unpublished data). Furthermore, they are facultative multi-brooded, allowing a within-individual comparison of egg size between subsequent clutches laid over the season under different conditions. A number of studies have looked into causes of this natural variation in egg size and found both temperature, laying date and food effects on egg size in great tits (Nager & van Noordwijk 1992; Nager & Zandt 1994; Nager et al. 1997; Christians 2002; Lessells et al. 2002; Pendlebury & Bryant 2005).

To disentangle the influences of food and temperature on egg size, as well as investigate the variation between females, we studied egg size of individual pairs of great tits under *ad libitum* food conditions in climate-controlled aviaries with a natural increase in photoperiod. We examined the variation in egg size between females, but also between clutches and individual eggs laid by the same female. We determined

the variation under standardized conditions and compare this variation to patterns found under natural, energetically limited, conditions in the natural population at the Hoge Veluwe National Park (The Netherlands), the same population where our captive birds originate from.

Predictions

If variation in egg size mainly results from food limitation, we expect birds with access to unlimited food and thus lifted energetic constraints to lay uniformly larger eggs than in the natural population. Contrary to this, if the observed natural variation is still present in captivity, then we need to identify the causes. Under *ad libitum* food conditions, variation might come about by specific female properties (genetically determined between-female variation in condition), environmental properties (seasonal changes in day length or temperature), or a combination of both. Furthermore, using related individuals, it is possible to explore a genetic component of egg size, causing sisters, which were hand-raised under standardized conditions, to lay similar-sized eggs compared to unrelated individuals.

We investigated effects of clutch number and position in the laying sequence on egg size and also compared effects of laying date of individual eggs. Both optimal egg size for the chick and optimal egg size for the mother will most likely vary seasonally in nature: early in the season, egg production costs are assumed to be higher for the female due to lower food availability and temperatures (but see te Marvelde et al. 2012). From the perspective of the chick, the risks of pre-hatching death in unfavourable, cold conditions is elevated for chicks born in smaller eggs early in the season (Krist 2011), but on the other hand the danger of starvation for early-born hatchlings might be lower as they are better synchronized with the seasonal food peak (te Marvelde et al. 2011). When we eliminate food constraints, we therefore expect the female to lay larger eggs early in the season only under very cold conditions when the risk of egg mortality is high, or if she invests unproportionally more in the first clutch independent of environmental condition. In contrast, we expect the female to increase egg size seasonally if the optimal egg size accounts for an increased starvation risk for late-born chicks. In addition, we expect within-clutch variation to be small to reduce sibling competition among chicks.

We investigated two female properties that might explain between-female variation: female size (measured as tarsus length) and the laying date of the female's mother in the wild. As all females were first-year breeders, age was not included in the analysis. All birds were expected to be equally well-fed, so we did not include female weight, fat score or other measures of body condition in our analysis. We might expect a positive correlation between female size and egg size, however, female mass, size or condition (e.g. mass standardized by size) was positively correlated with egg size in only 33 of

50 studies reviewed by Christians (2002) and the effect was inconsistent even within species. We also investigated genetic differences between early- and late-laying families by including the laying date of the female's mother in the wild. As we expect females originating from an early brood to be early layers themselves, and we expect early-laid eggs to be larger under *ad libitum* food conditions, we expect a negative effect of the mother's laying date on egg size.

To identify a relationship between temperature and egg size, we chose three partly overlapping and therefore correlated temperature periods. We investigated whether mean temperatures averaged over one, four and seven days pre-laying of an individual egg explained egg size. In great tits, the phase of rapid yolk formation is thought to take 3-4 days (Perrins 1970). We therefore chose a temperature period reflecting a) an immediate reaction to the temperature the bird was exposed to on the day before laying, which might most likely cause differences in the final amount of albumen deposited into the egg, b) a reaction to the temperature experienced over the complete period of rapid yolk formation, but also c) a slightly more long-term temperature influence, which might indicate systemic temperature effects on the entire reproductive physiology. In nature, ambient temperature is positively correlated with egg size in many species (reviewed by Christians 2002), but it is not known if this is mainly due to an effect on energy balances, or a direct effect *per se*. We therefore expect to find a positive relationship in case temperature does influence egg size through female energy metabolism even under *ad libitum* conditions. We expect a negative relationship in case females flexibly determine egg size to fit their environment, thereby increasing egg size in colder conditions to enhance thermoregulatory properties of the egg and provide more resources to chicks in harsher conditions.

Materials and Methods

Captive birds

Eggs from 132 captive first-year great tit breeding pairs were measured between 2006 and 2010. The birds were the offspring of known wild parents from a long-term monitored population at the Hoge Veluwe National Park (the Netherlands), which is also the population used for the comparison between captive and wild great tits. Offspring from 50 different wild families were used, and half of these families were specifically selected because they were early-breeding birds, whereas the other half were late-breeding birds. On day 10 post-hatching, chicks were taken to captivity at the Netherlands Institute of Ecology (Heteren) as complete broods for hand-raising under standardized conditions (Drent et al. 2003). After independence, fledglings were transferred to single-sex groups in open outdoor aviaries (2 x 4 x 2.5 m), where they were housed until December. Each year, 36 breeding pairs were formed randomly

within the early- or late-breeding families, avoiding sib-matings. Due to fatalities in the young birds, we formed some pairs by using 13 additional spare birds (including 6 females from 4 families) over 5 years, which were hand-raised in the same fashion. On the 1st of December the pairs were placed in climate-controlled aviaries to breed in the next year.

Aviary conditions

Breeding pairs were housed in 36 separate indoor aviaries (2 x 2 x 2.25 m) under a light regime mimicking the natural photoperiod, which was adapted twice weekly (i.e. for 52°N increasing from 7.45L(light):16.15D(dark) at the winter solstice to 16.30L:7.30D at the summer solstice). Light sources were three high frequency fluorescent light tubes, complemented with an 8 W bulb providing an additional half hour of dawn and dusk. A shaft from the roof (SolaTube), whose opening was synchronized with the light schedule, allowed for supplementary daylight. The birds were fed *ad libitum* with a constant daily amount of food, consisting of a mixture of minced beef, proteins and vitamin and mineral supplements (Nekton S and Nekton Bio, NEKTON GmbH, Pforzheim), completed by sunflower seeds, fat balls, a mix of dried insects (Carnizoo, Kiezebrink International, Putten), extra calcium and water for drinking and bathing.

Temperature treatments

Over five experimental years, birds were exposed to varying temperature regimes. Each season, a different experimental setup of two (2006 and 2007) or four (2008-2010) temperature treatments was used, each treatment being replicated in a regular design. For a rationale and thorough description of temperature treatments, see Visser et al. (2011a) and Schaper et al. (2012). Temperature treatments affected the onset of laying in 2008 and 2010, but not in other years.

In 2006 and 2007, the 36 pairs of great tits were divided into two groups that differed in the ambient temperature to which they were exposed, with the high temperature treatment set to be always 4°C higher than the cold temperature. From 1st December to the end of February temperatures were kept constant at 4 and 8°C, respectively, after which temperatures gradually increased by 0.65°C per week up to 1st July, reaching 15 and 19°C, respectively. This setup was repeated in the two years.

In 2008, all 36 pairs were exposed to a constant temperature of 15°C from December onwards until summer. In three groups, this temperature was lowered to 7°C in either February, March or April for a month, before it was increased to 15°C again, except for the latest cold period (April), which was maintained until the female initiated laying under cold conditions.

In 2009, there was no seasonal temperature pattern, but a temperature change over the day. Each treatment was composed of a high or low mean with either a high or low day-night amplitude. The two warm treatments were fluctuating around a mean of 14°C (11-17°C, high amplitude, or 13-15°C, low amplitude), while the two cold treatments were fluctuating around 8°C (5-11°C, high amplitude, or 7-9°C, low amplitude). For this year, effects of temperature variation on egg size were specifically addressed in a separate analysis.

In 2010, the setup of the experiment combined two consecutive temperature rises, one during early gonadal development, the other shortly before breeding. All birds were kept at 6°C from December until February. On 8th February, the first two groups experienced a rapid increase in temperature from 6 to 16°C over a course of two weeks, then stayed at 16°C for three or five weeks. On 15th or 29th March, respectively, temperature was increased to 20°C and stayed high during egg laying and moult. Starting on 22nd February, the other two groups were exposed to a more gradual increase in temperature from 6 to 11°C over a course of two weeks, thus experiencing a lower increase rate. These groups then stayed at 11°C for one or three weeks. On 15th or 29th March, respectively, temperatures increased to 15°C for egg laying and moult. Superimposed on the temperature profiles was a day-night rhythm of ± 1°C.

Data collection

Birds could choose between two nest boxes for breeding, which were inspected for eggs from outside the aviary without disturbance. Nesting material, consisting of moss and dog hair, was provided from March onwards. After nest building was observed, nest boxes were checked daily for eggs. The day that an egg was found is referred to as the laying date. Eggs were removed from the nest on the morning they were laid and replaced by eggs taken from nests of wild great tits used in cross-fostering experiments. Fresh egg weight was measured on a scale (Sartorius Basic BA 160 P, accuracy 0.0001 g) and length and width determined with a digital calliper (accuracy 0.01 mm). Egg volume was determined using Hoyt's equation (Hoyt 1979): $V=\pi/6LB^2$, where L is length, B is breadth. Nests and foster eggs were removed completely after five days of incubation, after which some females rebuilt and initiated new clutches. In cases in which females did not start to incubate after clutch completion, the foster-eggs were removed after 10 days in which no new egg was added to the clutch and the clutch was considered deserted.

Statistics

There was a tight correlation between fresh egg mass and egg volume ($r^2=0.91$). In the following analyses, egg weight was chosen as a measure of egg size for the captive birds, as it was measured with higher accuracy. Egg weight was analyzed with mixed

models (procedure lmer, package lme4, R 2.10.0, R Development Core Team 2009). Fixed effects were year, clutch number, position in laying sequence (continuous over all clutches), laying date, laying date2, mean temperature averaged over 1, 4 and 7 days pre-laying, female tarsus length and laying date of the female's mother. Random effects were female identity nested in family. In the final model the interaction between temperature and laying date was also tested. In an additional analysis, the effect of daily temperature variation in 2009 on egg size was tested by replacing mean temperature by temperature variation (high or low amplitude). Non-significant effects were eliminated in a stepwise model reduction procedure. P-values were calculated by Markov Chain Monte Carlo sampling (procedure pvals.fnc, package languageR). The results include Bayesian 95% highest posterior density credible intervals, equivalent to 95% confidence intervals. As year is given as a multi-level fixed factor, a P-value is created for every level in comparison to 2006.

Comparison with the natural population

The variation in egg size found under controlled conditions was compared with the variation found in the field population at the Hoge Veluwe National Park in the years 2008 and 2010, when a large proportion of wild great tit clutches were measured. The Hoge Veluwe contains about 400 nest boxes distributed over a 171 ha mixed woodland area on poor sandy soil. The vegetation is dominated by pedunculate oak (*Quercus robur*), Northern red oak (*Q. rubra*), larch (*Larix decidua*) and pine (*Pinus sylvestris*). After nest building started, nest boxes were checked daily to determine exact laying dates. Eggs were numbered on the day they were laid, but measured after clutch completion. As egg weight changes over time due to water loss, egg volume, calculated from length and width as described before, was used in the analyses. 168 great tit clutches were measured. Of these, only 6 nests were second or replacement clutches (after failure of the first clutch). Most clutches were measured completely, but in a small number of nests, egg sizes of the clutch were only assessed partly. Daily mean temperatures were retrieved from the Royal Netherlands Meteorological Institute (KNMI) station at Deelen, about 2 km from the field site.

Egg data from wild birds was analysed using a similar model as for the captive birds. However, family was not included in the model as too few females had family relationships. As the majority of clutches were first clutches, clutch number was not included in the model. Female age, tarsus length or laying date of the female's mother were also not accounted for.

To compare the variability in egg size in captive birds to the variability found in the Hoge Veluwe population, variance components were calculated for both datasets using a mixed model with female identity as a random factor. For the eggs laid by captive birds, this was done for a reduced dataset that only included first clutches and only eggs laid in a temperature range below 16°C to facilitate a better comparison

to the range experienced by wild birds, where only few eggs were laid above a mean temperature of 16°C. In addition, to assess the within-clutch, within- and between-female variance in eggs laid by captive birds, a mixed model containing a random effect of clutch nested in female, nested in family was used.

Results

a) Egg size variation in captivity

Over five years, 1657 eggs were measured from 247 clutches laid in the climate-controlled aviaries. These clutches were laid by 127 captive females, originating from 53 families. Females laid on average 2, but between 1 and 5 clutches. As eggs were removed after 5 days of incubation, these clutches sometimes followed each other closely in time and should be perceived as replacement clutches. Mean clutch size was 6.7 eggs. Each female laid on average 11 eggs over subsequent clutches, with a minimum of 1 and a maximum of 44 eggs. These eggs were laid between the 7th April and the 19th July. Mean laying date of all clutches was the 20th April. Mean egg size, measured as fresh egg weight, was 1.61 g.

Eggs were on average larger in 2006 and especially large in 2010, compared to 2007-2009 (Table 7.1). Mean egg weight did not change over the laying sequence (over all clutches) and was similar for first and replacement clutches (Table 7.1). Egg size first increased in early April, but then stayed constant over time, represented by a quadratic date effect (Table 7.1, Fig. 7.1A).

There was a slight, but significant, seasonal increase in experimental temperatures for all years taken together (r^2=0.09), even though in years 2008 and 2009 daily mean temperatures did not increase seasonally. The temperature period best correlated with egg size was the period of 7 days pre-laying. Over the experienced range between 8 and 21°C females laid slightly larger eggs in colder conditions than in warmer conditions (Table 7.1, Fig 7.1A). Egg weight thus decreased by 0.02 g for every 1°C temperature increase, which means an average reduction in egg weight by 0.2 g over the experienced temperature range. An interaction between temperature and laying date effects on egg size was not significant (t=-0.58, P=0.56). Differences in egg size could not be attributed to female body size (tarsus length), or the laying-date of the female's mother (Table 7.1). An analysis based on egg volume instead of egg weight yielded qualitatively similar results.

When re-analysing the dataset for a range comparable to the natural population, thus restricting it to temperatures below 16°C and first clutches laid, the negative relationship between temperature pre-laying and egg size persisted (t=-3.47, P<0.001). However, the quadratic date effect was dropped from the model due to the fact that late laying dates have been eliminated by the restriction to first clutches.

Table 7.1: Model results for the analysis of egg size (analysed as egg weight) in climate-controlled aviaries in the years 2006 to 2010. Explanatory variables include three mean temperatures, averaged over 1, 4 and 7 days pre-laying. 'Laying date female's mother' denotes the day that the first egg of the clutch that the female originates from was laid in the wild. Female identity, nested in female family, is fitted as a random effect. The results are presented including lower and upper Bayesian 95% highest posterior density credible intervals (L 95% HPD, U 95% HPD). As year is given as a multi-level fixed factor, a P-value is created for every level compared to 2006.

Explanatory variable	Estimate	L 95% HPD	U 95% HPD	t	df	P
Clutch	-0.018	-0.023	0.016	-1.69	1	0.091
Position in laying sequence	-0.0001	-0.0006	0.0040	-0.05	1	0.96
Laying date	0.0088	0.0061	0.010	8.60	1	**<0.001**
Laying date^2	-0.00005	-0.0001	-0.00003	-5.37	1	**<0.001**
Mean temperature 1 day pre-laying	-0.0029	-0.019	0.016	-0.34	1	0.73
Mean temperature 4 days pre-laying	0.022	-0.010	0.049	1.536	1	0.12
Mean temperature 7 days pre-laying	-0.022	-0.024	-0.012	-6.41	1	**<0.001**
Laying date female's mother	-0.0024	-0.0066	-0.0001	-0.95	1	0.34
Tarsus length female	-0.0003	-0.0038	0.0034	-0.10	1	0.92
Year 2007	-0.14	-0.19	-0.072	-3.17	1	**0.002**
Year 2008	-0.14	-0.20	-0.072	-3.06	1	**0.002**
Year 2009	-0.17	-0.22	-0.099	-3.93	1	**<0.001**
Year 2010	0.15	0.070	0.21	2.92	1	**0.004**

b) Egg size variation in wild birds

In 2008 and 2010, 1507 eggs were measured from 167 females, and only one female was measured in both years. Mean clutch size for all clutches in the Hoge Veluwe population, including nests in which eggs were not measured, was 9 eggs in these years, ranging from 1 to 15 eggs. Mean egg size was 1.59 cm^3. In 2008, mean laying date was 26th April, in 2010 19th April, but egg size did not differ between years (Table 7.2).

Egg size increased over the laying sequence within the clutch, rather than over time (Table 7.2, Fig. 7.1B). There was no date effect when the laying date of the first egg was added to the model besides the position of the egg in the laying sequence. The temperature period that best predicted egg size under natural conditions was the period 4 days pre-laying: When temperatures in this period were warmer, females laid slightly larger eggs (Table 7.2, Fig. 7.1B). Egg volume increased by 0.004 cm^3 for every 1°C temperature increase, which means an average increase by 0.06 cm^3 over the experienced range between 4.5 and 18°C. Furthermore, temperature had no quadratic effect on egg size (t=-1.88, P=0.061).

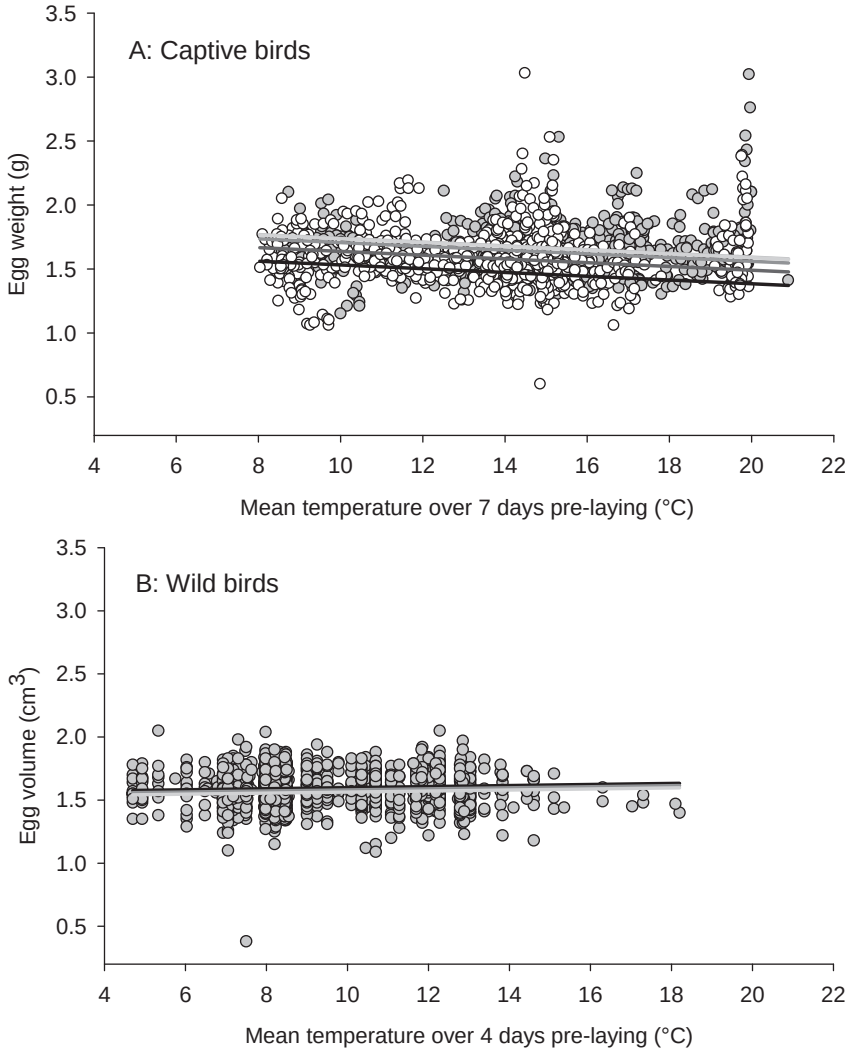

Figure 7.1: Effects of temperature, laying date and position in the laying sequence on egg size of great tits. **A: Captive birds**: Fresh weight of eggs laid by pairs of captive great tits in climate-controlled aviaries. Birds slightly increased egg size if mean temperatures averaged over 7 days pre-laying were colder. Eggs laid in first clutches (open dots) did not differ in size from eggs laid in subsequent replacement clutches (grey dots), even though egg size first increased seasonally and then stayed constant over time. Lines represent model estimates for laying dates 20, 40, 60, 80 and 100 April (1=1st April). Line colours get lighter as laying dates get later. Lines for laying dates 80 and 100 are superimposed. **B: Wild birds**: Volume of eggs laid by pairs of wild great tit. Birds slightly increased egg size if mean temperatures averaged over 4 days pre-laying were warmer. Egg size also increased over the laying sequence. Lines represent model estimates for egg number 2, 6 and 10 of a clutch. Line colours get lighter with egg number.

Table 7.2: Model results for the analysis of egg size (analysed as egg volume) in the wild population at the Hoge Veluwe, of which the captive birds originate from, in the years 2008 and 2010. Explanatory variables include three mean temperatures, averaged over 1, 4 and 7 days pre-laying. Female identity is fitted as a random effect. The results are presented including lower and upper Bayesian 95% highest posterior density credible intervals (L 95% HPD, U 95% HPD). In comparison to the analysis of eggs laid by captive birds, the dataset is mostly formed by first clutches, therefore clutch is not a part of the model. Laying date of the female's mother, as well as female tarsus length are also not included in this model.

Explanatory variable	Estimate	L 95% HPD	U 95% HPD	t	df	P
Position in laying sequence	-0.0041	-0.0055	-0.0019	-4.68	1	**<0.001**
Laying date	-0.0005	-0.0026	0.0007	-0.54	1	0.59
Laying date2	-0.00002	-0.0001	0.0001	-0.35	1	0.72
Mean temperature 1 day pre-laying	-0.0007	-0.0036	0.0018	-0.53	1	0.60
Mean temperature 4 days pre-laying	0.0042	0.0004	0.0064	2.70	1	**0.0071**
Mean temperature 7 days pre-laying	-0.0062	-0.014	-0.0006	-1.85	1	0.064
Year 2010	-0.016	-0.036	0.010	-1.05	1	0.29

c) Comparison between captive and wild birds

When comparing the variation in egg size from females breeding in climate-controlled aviaries under *ad libitum* conditions and under natural conditions, it was apparent that the total variance in egg size was twice as large in captivity (Table 7.3), even though the analysis excluded eggs laid under high temperatures in captivity. The range of egg sizes included extreme values of 0.6 g and 3.0 g in captive females, whereas wild females laid eggs between extremes of 0.4 cm^3 and 2.1 cm^3 (Fig. 7.1A,B). As an egg volume of 1 cm^3 correlated with a weight of 0.93 g for eggs laid by captive females, this means that wild females laid eggs between 0.37 g and 1.95 g. A large part of this variation was in both cases explained by differences between females and in captivity between-female differences explained slightly more variance: 60.8% for captive females compared to 54.2% for wild females (Table 7.3). When analysing the complete dataset of all eggs laid in captivity, the total variance increases by including eggs laid under higher temperatures and in replacement clutches (Table 7.3). In the complete dataset, most of the variation can be explained by between-female, rather than within-female differences accounted for by between-clutch variation (Table 7.3, Fig. 7.2), but egg size also varies consistently between families (Table 7.3, Fig. 7.3).

To further explore how much of the temperature effect found in captive birds was due to between-female, rather than within-female variation, we performed a within-subject centering procedure (van de Pol & Wright 2009). Egg size was standardized by substracting the mean egg size per female from the size of each individual egg and adding mean egg size per female as a fixed factor to the model. In this model, standardized egg size was not affected by temperature (t=-1.45, P=0.15).

Table 7.3: Variance components of random effects for egg size are given for three datasets. (a) variance in egg size explained by clutch, nested in female, nested in family, was analysed for captive birds in climate-controlled aviaries (n=1657 eggs). (b) variance in egg size explained by female was analysed for the captive birds, restricted to eggs from first clutches laid over a temperature range comparable to the wild population (mean temperatures a week pre-laying 8-16°C), for reasons of comparability (n=870 eggs). (c) variance in egg size explained by female was analysed for wild birds, predominantly laid in first clutches over a temperature range between 6 and 16°C (n=1507 eggs). Variance is also given as percentage of total variance. SD=standard deviation.

Dataset	Random effect	Variance	Variance (%)	SD
a) Aviaries, full dataset	Clutch	0.006	14.6	0.08
	Female	0.014	31.8	0.12
	Family	0.011	24.2	0.10
	Residual	0.013	29.4	0.11
	Total	0.044	100	
b) Aviaries, restricted dataset	Female	0.022	60.8	0.15
	Residual	0.014	39.2	0.12
	Total	0.036	100	
c) Hoge Veluwe population	Female	0.010	54.2	0.10
	Residual	0.008	45.8	0.09
	Total	0.018	100	

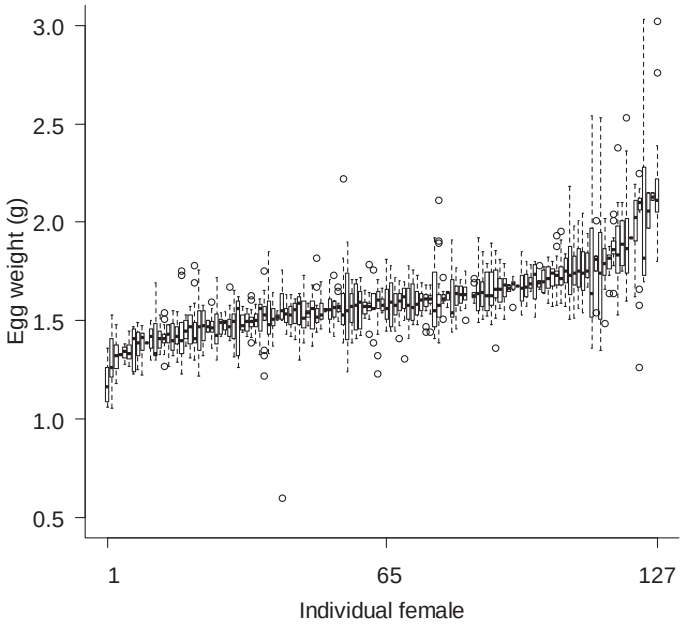

Figure 7.2: Between-female variation in egg size in captive great tits. Boxplots showing the variability in egg size (measured as fresh egg weight) for individual great tit females kept in climate-controlled aviaries.

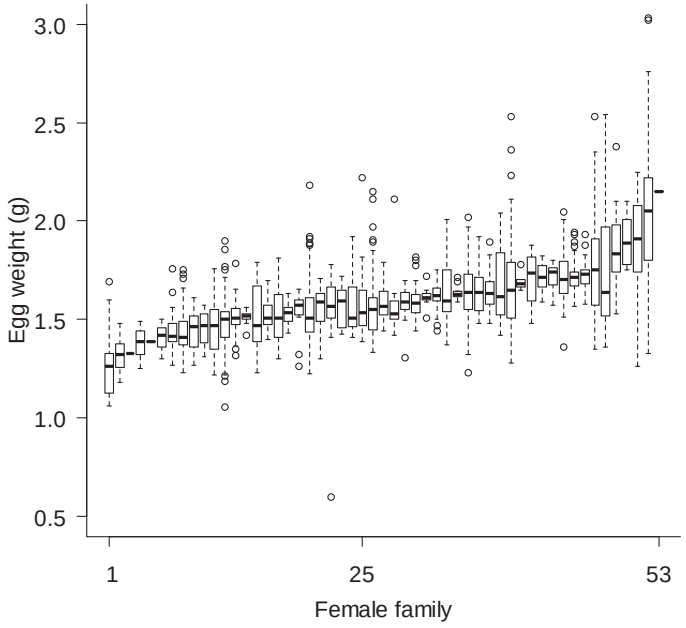

Figure 7.3: Between-family variation in egg size in captive great tits. Boxplots showing the variability in egg size (measured as fresh egg weight) per family for great tit females kept in climate-controlled aviaries.

d) Causes of extremely large egg size in captive birds

To investigate factors that could be responsible for the extremes in egg size by captive females, the 20 largest eggs in the dataset (weighing more than 2.18 g) were investigated. Huge eggs were laid in years 2006, 2009 and mostly in 2010. In 2010, 16 huge eggs were laid by 6 females originating from 5 different families. These eggs were laid in warm conditions, over a temperature range above 11°C, but mostly around 20°C. They appeared in various positions in the laying order, even though some females stopped egg laying after having laid one to three huge eggs last. Huge eggs were interspersed with normally-sized eggs, but seldom laid in sequence. They were not laid on specific dates or after any noticeable events. Sometimes, but not always, they followed a laying gap the day before. In summary, none of the examined factors can explain why several females from different families and in different years laid eggs of a size normally not found under natural conditions.

Discussion

Egg size variation

We investigated direct effects of temperature on egg size under conditions of unlimited food but variable temperature in birds kept in climate-controlled aviaries. Egg size, measured as fresh egg weight, was far more variable than in a natural population also reported here. This variability also exceeds by far the variation reported for other wild populations: Perrins (1970) measured egg sizes for more than 4500 great tit clutches in a British population. The smallest eggs which produced surviving young weighed 1.0 g, the largest 2.1 g. Van Noordwijk et al. (1981) reported a range between 1.3 g and 2.0 g in four Dutch great tit populations, including the Hoge Veluwe population. This also compares to data reviewed by Christians (2002), who reports that the largest egg is normally not larger than twice the size of the smallest egg in a range of bird species and generally only about 50% bigger. Why do eggs laid in captivity thus show such a large variation in size, mostly diverging towards larger eggs? Females in the aviaries were hand-raised from the nestling stage onwards and are thus not exposed to natural selection, meaning that also low-quality individuals are present in the dataset. However, if the larger variation found in captivity would be caused by the missing selection for better-quality-females, we would expect a higher variability at the lower egg size range, while in the present case especially the presence of larger eggs is intriguing. In principle, this effect could be explained by the presence of *ad libitum* food. However, in a natural context, food supplementation increased egg size in only 36% of studies on various avian species, but never more than 13% (Christians 2002). In great tits, Nager et al. (1997), found that food supplementation with either protein-rich mealworms or protein-poor sunflower seeds did not significantly increase egg size in wild birds. Enriching the diet of wild birds thus seems not to increase egg size substantially. In contrast, it seems possible that a sheltered captive environment, where females have access to overabundant food and do not have to forage extensively, allows some, but not all females to lay exceptionally large eggs, so that variation in egg size exceeds 150% of the mean. We expected egg size to be uniformly larger in our experiment, which is generally not the case, indicating that not energy-limitation alone drives variation in egg size. This finding is also supported by Krist (2011), who found that egg size-variation was generally larger in captive compare to wild bird populations. In the following, we further explore causes of the large variability between and within females in more detail.

Temperature effects on egg size

Egg size was affected by temperature, but surprisingly and in contrast to observations in the field higher temperatures decreased egg size slightly. Ambient temperature can directly and indirectly influence daily energy expenditure during egg laying by affecting thermoregulatory costs and foraging behaviour (Stevenson & Bryant 2000;

te Marvelde et al. 2012) and was repeatedly shown to lead to smaller clutch sizes and/or smaller eggs in various wild bird species (Christians 2002), including the great tit (Nager & van Noordwijk 1992; Nager & Zandt 1994; Lessells et al. 2002). Also in the Hoge Veluwe population investigated here, which is the source population of all individuals used in the aviary experiment, a slight, but positive relationship between egg size and ambient temperature was found. However, under *ad libitum* food conditions temperature seems to not affect egg size via energy limitation any more. The negative relationship between temperature and egg size could be explained by two factors: First, egg size also affects the surface area, as the volume ratio decreases as egg size increases, so that larger egg should retain heat better (Williams 1994). For a female that is not energy-limited it would therefore seem advantageous to lay larger eggs under colder conditions to maintain a more stable temperature environment. Second, independent of laying date, positive effects of egg size on the hatching chick are more apparent in harsh, low-quality environments (Christians 2002) and therefore laying larger eggs in cold springs, where both food availability for chicks is lower and thermoregulatory costs are higher would lead to a higher increase in fitness. As egg size mostly affects immediate survival over the first week, and in less harsh, or warmer, environments, parental care can probably compensate for a less-than-optimal start (Christians 2002). Our results indeed indicate a female tendency to improve the chick's egg environment under cold conditions if she is not restricted by energy. However, even under controlled conditions this effect is small compared to the overall, mostly between-female, variation in egg size. Apparently egg size is more variable above 15°C due to some individuals laying extremely large eggs (>2.5g). Phenotypic plasticity in egg size in response to temperature is thus only a small part of the story. There are substantial between-female differences in egg size in response to temperature. This is to a large part due to the fact that individual females were exposed to very different experimental temperatures, and some individuals were laying all their eggs under almost constant temperature conditions. For these females, it is not possible to determine a true reaction norm towards temperature. However, by visually examining the relationship between temperature and egg size for all females individually, we found that most females experiencing a wide range of temperatures while laying decreased their egg size with temperature, thus showing the plasticity that is also indicated by our analysis.

In our experimental setup, averaged temperatures over 7 days seemed to predict egg size better than temperatures in the final period of egg development in which the egg is matured and the outer albumin layers added. This is in accordance with a study by Stevenson & Bryant (2000), who investigated influences of natural temperature on egg size in great tits. The authors progressively lengthened the temperature-averaging period from one day up to nine days and even though in their natural setup temperature had a positive influence on egg size, they also found the residual variance to be lowest when taking into account an eight-day temperature average pre-laying.

However, in the present study, an averaging over a period of four days pre-laying was more appropriate under natural conditions. Lessells et al. (2002) found that neither albumen, shell nor yolk weights of wild great tit eggs increased with temperature averaged over three days pre-laying. Taken together, these results indicate that egg size does not only depend on the nutrient availability in the day(s) pre-laying, which is at least partly determined by ambient temperature in a natural setup (Avery & Krebs 1984; Valtonen et al. 2011). The results obtained using captive birds seem to imply that females are principally selected to invest more in eggs under harsher conditions in the long run. On the other hand, our finding that under controlled conditions a temperature averaged over a longer period was better correlated with egg size could also mirror the lower variability in experimental temperatures compared to the natural situation, rather than any biological relevance.

We also assessed whether temperature variation affected the ability of a female to lay large eggs. We tested this by comparing eggs laid by females in different temperature treatments, which, independent of the mean temperature, showed a high or low temperature amplitude over the day. Pendlebury & Bryant (2005) found that egg mass of great tits tended to be smaller if ambient temperatures during one week pre-laying were more variable (controlled for mean temperature). In their study, temperature variability accounted for 4% of variation in egg mass, compared to 4.7% accounted for by mean temperature alone, which had a positive effect on egg size. They suggested that metabolic rates increase with higher daily temperature ranges and rapid sinusoidal temperature fluctuations (Prinzinger 1982; Pendlebury et al. 2004), and therefore a higher variation would lead to higher energetic constraints. Mean temperatures in their study ranged between 6 and 16°C, while mean temperatures for the 7-day period showed a daily variance of 0.05 to 2.3°C. In our experiment, variance over a day was either set to be 2 or 6°C in 2009, but stayed constant over time. As this large difference in temperature fluctuations did not affect egg sizes, we cannot find support for an effect of moderate temperature fluctuations on metabolic rates, resulting in different egg sizes.

Seasonal effects on egg size

We found a seasonal pattern in egg sizes that mirrors the situation encountered in the field. Also under controlled conditions, egg size first increased, then stayed constant over the late season. Clutch number, or position in the laying sequence, in contrast, had no additional effect on egg size, whereas under natural conditions position in the laying sequence, rather than date, influenced egg size positively. It is important to keep in mind that the birds in the current setup were re-laying up to four times after a completed clutch was taken away from them, and so it is not straightforward to compare 'clutch effects' to a natural setup, where second clutches are laid only after the first brood has left the nest. Perrins (1996) found that in a natural population

of great tits eggs were smaller at the beginning of the season (when temperature were naturally lower). Adding temperature significantly reduced the influence of laying date in their model, but did not eliminate it, suggesting an underlying date-effect that is independent of energy-limitation. In contrast, Nager & Zandt (1994) found that relative egg-size in great tits (corrected for female size) did not differ with laying date, but females laid larger eggs in repeat clutches compared to first clutches, which appear earlier in the season. Christians (2002) found in his extensive review that approximately 70% of the variation in egg size within species was indeed due to variation between rather than within clutches, a hypothesis we cannot test in our wild population, as we are restricted to look at mostly first clutches. Most studies indeed found no direct relationship between egg size and laying date, but if it was present, it was often negative (Christians 2002), similar to what we find under natural conditions.

Taken together, these results give some support to the fact that independent of temperature, there is a seasonal change in egg size that is not solely explained by energy-limitation due to increasing food availability, as it persists in captive birds. In our experiment, the date effect is unlikely due to an initial increase in 'efficiency' of the reproductive machinery, which would be more clearly reflected in size differences over the laying sequence. It could rather be explained by females being selected to give later-born chicks, which are born under deteriorating food conditions, a starting advantage in form of egg nutrients. Therefore, females may vary egg size in relation to laying date *per se* in order to anticipate seasonal changes in costs and benefits to nestlings of hatching from large eggs (Murton & Westwood 1977; Magrath 1992; Lessells et al. 2002).

Between-female variation and genetic effects

Under natural conditions, differences among great tit females are a major component of the variance in egg size (Ojanen et al. 1979), and egg size is usually highly repeatable among clutches of the same female (Boag & van Noordwijk 1987). Krist (2011) showed that the repeatability for egg size is usually larger than 0.6 in various avian species, and the heritability larger than 0.5 (however, because of common environment effects, this value might be an overestimation, Krist 2011). This high repeatability is suggested to be due to consistent or inherited properties, such as foraging skills, social dominance and territory quality, which all affect food supply and therefore individual condition. Nevertheless, that female condition determines egg size is refuted by the fact that egg size is commonly not related to other measures of quality, such as clutch size, timing of laying or the ability to rear young (Christians 2002). Krist (2011) reports that female mass, size and condition are positive correlated with egg size in 33 of 55 evaluated studies (and negatively correlated in only 2 cases). In general, female mass explained less than 20% of the egg size variation, but the effect

was inconsistent even within species. Other authors found a significant positive relationship between egg size and blood haemoglobin (r^2=0.18) or protein condition (Christians 2002), but so far no mechanism could identify this relationship, and so far no endocrinological technique (such as estradiol or follicle stimulating hormone treatment) has yet increased egg size (Christians 2002).

This shows how limited our knowledge of between-female variation in egg production still is even after extensive studies and how important experiments on related individuals under controlled conditions are in this context. In our experimental setting, traits related to energy or nutrient availability cannot explain the consistent differences between females, which are even larger under controlled than under natural conditions. Traits related to foraging efficiency, dominance or other quality measures can also not explain the observed resemblance between sisters under controlled conditions, if not the conversion of food to egg mass was greatly different between females, which we believe to be unlikely. So far we cannot explain between-female differences in egg size by using female-specific properties like size, or traits related to their propensity to lay early or late in the season. It is thus a challenge for the future to further investigate in what respect these females differ from each other in their genetic make-up.

Concluding remarks

Under natural conditions, avian egg size varies consistently between females, but also between individual eggs of the same female. The principal question is if this variability reflects different optima for individual females due to genetic differences in condition, changing optima over different contexts, or if it rather reflects nutritional or physiological limitations (Christians 2002). While variation between females is most often explained by quality differences, the variation between individual eggs of the same female is commonly linked to seasonality, both in food abundance and temperature. When investigating the effect of temperature on egg size under controlled conditions and *ad libitum* food supply, a seasonal increase was still apparent, as well as a negative influence of pre-laying temperature. The fact that low temperatures induce laying of larger eggs in birds that are not energy-limited could be explained by the fact that larger eggs have better thermoregulatory properties, and chicks hatching under colder conditions are more dependent on resources deposited into the egg to survive. That a positive influence of temperature on egg size did not prevail under captive conditions clearly shows that temperature only indirectly influences egg investments under natural conditions. Additionally, the similarity between related females shows a genetic effect influencing egg size that cannot be caused by foraging ability or social status. Egg size in individual females showed exceptional variation under controlled conditions. Maternal investments in individual eggs are therefore not merely a product of female condition, but a finely-regulated trait which greatly affects both female and offspring fitness alike.

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Chapter 8

Samuel P. Caro, Sonja V. Schaper, Gregory F. Ball and Marcel E. Visser

The unresolved causal influence of temperature on seasonal timing in homeotherms

Manuscript

Introduction

Ambient temperature varies in time and space on time-scales ranging from a few hours to months (seasonality), up to decades (long-term climate change). Homeothermic animals are therefore continuously challenged to regulate and maintain functional capacities and homeostasis as their thermal environment changes (Johnston & Bennett 1996). Although homeothermy has facilitated species to live in habitats with a wide range of environmental conditions, most birds and mammals still heavily depend on temperature for the temporal organization of their life-cycle. Seasonal timing, or phenology, is well pronounced in virtually all birds and mammals of the temperate zone and it enables organisms to synchronize the energy-intense stages of the life-cycle to a time of the year when environmental conditions are favourable (Bradshaw & Holzapfel 2008; Visser et al. 2010a). Life-cycle stages such as breeding, growth, migration or moult require such a high level of energy that they can only take place when ambient temperature is mild or when particular sources of energy, themselves dependent on temperature, are available. The timing of these favourable conditions, often the phenology of the food organisms, are determined by temperature patterns and hence also the yearly variation in the seasonal timing should be affected by temperature or by environmental variables closely associated with temperature (Visser 2008). There is ample evidence that seasonal timing in a wide range of organisms is correlated with ambient temperature (see below). But despite this clear evidence it is to a large extent unclear how temperature is causally linked to seasonal timing in birds and mammals.

Understanding the causal relationship between temperature and phenology in homeotherms is especially important as temperature is currently rapidly increasing due to global climate change. Consequences of this increase are apparent in societal, financial and environmental impacts in terms of changes in biodiversity, productivity and ecosystem services (IPCC Core Writing Team 2007a). Over the past three decades, one of the best-documented impacts of global warming on living organisms has been on phenology. Clear phenological shifts over time have been observed in many taxonomic groups in terrestrial, aquatic and marine environments (Parmesan & Yohe 2003; Root et al. 2003; Cleland et al. 2007; Parmesan 2007; Thackeray et al. 2010). However, the rate at which different species change their phenology is highly variable, sometimes causing momentous phenological mismatches in food chains (Visser & Holleman 2001; Post & Forchhammer 2008). These mismatches can influence population viability and biodiversity by affecting individual fitness (Both et al. 2006), making it necessary for species to adapt to global climate change.

In a warming world, organisms can adapt in different ways: they can make use of phenotypic plasticity to adapt their life-cycle, they can change genetically through micro-evolution, including changes in the degree of plasticity, or migrate to higher latitudes or altitudes to maintain their current phenology in a similar thermal

environment. However, the maximum rate at which organisms will be able to adapt to novel environmental conditions will primarily be set by the rate of micro-evolution of physiological traits underlying the timing of a life-cycle stage (Visser 2008). As all stages of an animal's life-cycle are orchestrated and regulated by underlying physiological mechanisms, the understanding of the evolutionary potential in these phenotypic traits will inevitably be closely linked to the detailed knowledge of the micro-evolution of physiological mechanisms that control their expression (Finch & Rose 1995; Brakefield et al. 2003; Lessells 2008; Lyon et al. 2008; Chown et al. 2010; Robinson et al. 2010). It is therefore the regulatory systems rather than the emerging timing traits that have been shaped by natural selection and that will have to evolve in response to a changing thermal environment (Sultan 2007). Understanding how these regulatory systems may evolve requires the integration of disciplines such as evolutionary ecology, physiology, endocrinology, neurobiology, molecular biology and genetics, which have often developed independently from each other (Visser et al. 2010a; Williams 2011).

Establishing the causal effect of temperature on phenology is critical if we want to predict the maximum rate of temperature increase organisms can cope with or adapt to in the coming decades (IPCC Core Writing Team 2007b). At present, this causal effect of temperature on seasonal timing and the underlying physiological basis of temperature integration in birds and mammals is still very poorly understood. Our knowledge about how moderate seasonal temperature changes are perceived at the physiological level to facilitate long-term predictions is very limited. Here, we want to integrate a mechanistic approach of temperature perception with insights from both natural observations and experimental studies about causal temperature effects on seasonal timing in homeotherms and provide a road map for an integrated research approach.

Correlational evidence

All around the globe and in temperate zones in particular the occurrence of phenological events such as flowering of plants, emergence of insects, timing of migration, breeding, moult or hibernation are strongly influenced by ambient temperature, with warm years generally corresponding with earlier phenology (Slagsvold 1976; Dhondt & Eyckerman 1979; Hutchings & Myers 1994; Fitter et al. 1995; Nager & van Noordwijk 1995; Reading 1998; Inouye et al. 2000; Visser & Holleman 2001; Dunn 2004; Thomas et al. 2010). In consequence, under climate change many phenological events tend to advance from year to year (Dunn & Winkler 1999; Fitter & Fitter 2002; Sanz 2002; Reale et al. 2003; Root et al. 2003; Møller 2008; Ozgul et al. 2010). Within the homeotherms much more evidence on correlations between temperature and seasonal timing is available for birds than for mammals. For example, a recent analysis of monitoring programmes of spring and summer phenology in the United

Kingdom included 84 species of birds, but only 4 species of mammals (Thackeray et al. 2010). Most evidence for effects of climate change on mammals involves range shifts (Burns et al. 2003; Moritz et al. 2008; Maiorano et al. 2011), only a few studies have investigated phenological shifts. While evidence is scarce for mammals, we know that birds' phenology is strongly influenced by temperature. Both migration and timing of breeding show year to year variation in response to temperature, with birds migrating and breeding earlier in warmer years (Perrins 1970; Slagsvold 1976; Perrins & McCleery 1989; Crick et al. 1997b; McCleery & Perrins 1998; Sokolov 2000; Jenni & Kery 2003; Both et al. 2004). There is, however, large variation among populations in the extend of this correlation, as not all populations shift their mean laying date with warming spring temperatures (Visser et al. 1998; Visser et al. 2003).

The few studies that have investigated the relationship between temperature and timing in mammals have produced inconsistent results, particularly when it comes to timing of reproduction, which, in contrast to birds, does not seem to be strongly influenced by temperature (Post & Forchhammer 2008). Mammalian gestation length is often long and inflexible, meaning that mammals need to use long-term temperature cues in prediction of conditions later on in the season when the young are reared. While yellow-bellied marmots (*Marmota flaviventris*) and red squirrels (*Tamiasciurus hudsonicus*) have been shown to advance their timing of breeding under climate warming (Reale et al. 2003; Ozgul et al. 2010), species with longer gestation length such as Japanese macaques (*Macaca fuscata*) (Gouzoules et al. 1981; Fooden & Aimi 2003), feral cattle (*Bos taurus*) (Burthe et al. 2011), caribous (*Rangifer tarandus*) (Post & Forchhammer 2008) or Alaskan moose (*Alces alces*) (Bowyer et al. 1998) do not seem to significantly adjust their breeding phenology to ambient temperature. Only red deer (*Cervus elaphus*) exhibited significant temporal trends in six phenological traits (oestrus date and parturition date in females, antler cast date, antler clean date, rut start date and rut end date in males) in response to climate warming (Moyes et al. 2011). Gestation length in red deer has also been suggested to become more variable in recent years (Asher 2007).

Evidence for a causal relationship

Demonstrating a causal effect of temperature on seasonal timing most often requires experiments under controlled conditions, which is not an easy task when using birds or mammals as study organisms (Peterson et al. 2010). A few studies in great tits (*Parus major*) have recently demonstrated a causal relationship between temperature and timing of egg laying (Meijer et al. 1999; Salvante et al. 2007; Visser et al. 2009). In addition, it has been shown that in contrast to previous beliefs, it is not the average temperature that matters for the birds when it comes to laying, but rather the pattern of temperature increase (Visser et al. 2011a; Schaper et al. 2012). Yet, temperature cues do not affect all life-cycle stages in the same way in birds, or it is not the same

component of temperature that affects these events (Schaper et al. 2012). For example, although the absolute mean temperature does not affect the onset of laying in birds, it strongly affects gonadal regression and the onset of moult in autumn, which has been experimentally demonstrated in various songbird species (Maney et al. 1999; Silverin et al. 2008; Dawson & Visser 2010). In addition, the same temperature cues have been shown to affect spring gonadal development in female, but not in male Puget Sound white-crowned sparrows (*Zonotrichia leucophrys pugetensis*) (Maney et al. 1999). In mammals, temperature does affect the timing of moult in the opposite way than in birds: warm temperatures delay the onset of the moult in autumn (Lynch 1973), while they advance the onset of moult in spring (Rust 1962). This is possibly attributable to the fact that the coat also provides an important means of insulation and the timing of coat change is thus directly adjusted to temperature changes in mammals, while feathers are mostly replaced once a year to maintain structural integrity and enable flight. In birds the energy-intense replacement of worn feathers is thus timed to conditions of high food abundance and, if anything, occurs earlier in a warmer year (Dawson 2005a; Dawson & Sharp 2010). Obviously, the scarcity of experimental approaches investigating temperature effects on seasonal timing, especially in mammals, is at the moment hampering our understanding of this relationship.

Mechanistic approaches

Although it has never been a forefront topic in avian and mammalian physiology, there have been a few studies on the effect of ambient temperature on physiological trait regulation. However, as the causal link between temperature and most phenological events only starts to be unravelled, the physiological traits that have been used could, at best, be considered as potential proxies for the phenological events of interest. As an example, most studies involving temperature manipulations in birds have looked at the effect of temperature on gonadal growth and the arising evidence is mixed (Wingfield et al. 1996; Wingfield et al. 1997; Maney et al. 1999; Wingfield et al. 2003; Dawson 2005a; Silverin et al. 2008; Schaper et al. 2012). This might not be surprising as most of these studies have also used constant temperatures which, as we now know for birds at least, is not relevant for their timing of breeding (Schaper et al. 2012). To complicate things even further, when temperature is found to affect timing, it does not always affect the proxies used, such as gonadal growth (Schaper et al. 2012). We therefore urgently need a better comprehension of the rules that drive temperature perception, transduction and integration on physiological traits that matter in a context of seasonal timing. Thermoreception has mostly been studied with regards to thermoregulation in homeoherms. In general, the neural organization of thermoregulation can be divided into a) thermoreception and afferent neural pathways, b) integration of thermal inputs in the central nervous system and c) effector pathways for autonomic and behavioural regulation. In the framework of temperature effects on seasonal timing, we can profit from these insights, which are mainly based on mammalian studies.

A) Temperature perception

Temperature is not sensed by specialised sensory organs but by thermoreceptors, which are non-specialized nerve endings of sensory neurons. There are distinct populations of these sensory neurons in the skin and they are selectively activated upon exposure to heat, non-painful warmth, non-painful cold, or painfully cold temperatures. Temperature and pain receptors are thus closely related. The thermal response properties and expression patterns of these populations of neurons vary considerably and account for a wide range of temperatures. More specifically, thermoreceptors are ion channels belonging to the transient receptor potential (TRP) superfamily. These channels are activated by a large diversity of stimuli such as temperature, pressure or chemical agents (Dhaka et al. 2006; Caterina 2007). The molecular makeup of thermosensation is highly conserved across taxa, including specialization of TRP channel function over different temperature ranges (Caterina 2007). Even non-vertebrates possess TRP channel isoforms that participate in sensory functions (Voets et al. 2005). Most TRP receptors studied so far are activated by relatively high temperatures (e.g. TRPV1-4 are activated by temperatures ranging from 27 to 52°C) and are therefore not relevant for the perception of fluctuations of spring ambient temperatures in the temperate zone. TRPM8, however, is considered as a cold receptor. It is expressed in a distinct subset of sensory neurons that is activated once temperature drops below 26°C, showing that in homeotherms this channel plays a relevant role in the detection of environmental temperature rather than body temperature (Peier et al. 2002; Myers et al. 2009). Mice lacking the TRPM8 gene are severely impaired in their ability to detect cold temperatures (Daniels & McKemy 2007). Similarly, TRPA1 (ANKTM1) has an activation threshold around 17°C (Story et al. 2003). Both in mammals and birds, it has been suggested that cold receptors are more abundant than warm receptors, but cold receptors seem less sensitive in birds than in mammals (Schäfer et al. 1988; Schäfer et al. 1989), which calls for more work on their discovery and characterisation of cold receptors.

In birds, epidermal thermoreceptors are concentrated in unfeathered parts of the skin, beak and tongue (Necker 1972; Necker 1973; Necker 1977; Necker & Reiner 1980; Gentle 1987; Gentle 1989; Schäfer et al. 1989; Dawson & Whittow 2000). In mammals they are more evenly distributed, but again high concentrations of thermoreceptors occur in the facial skin (Schäfer et al. 1988). Interestingly, studies describing thermoreception in birds were performed in chickens and pigeons, species that are less seasonal than most other avian species. We might therefore expect different spatial and density patterns and temperature sensitivities of thermoreceptors in more seasonal avian species. As most of the research effort has been conducted in the context of thermoregulatory function or pain sensation, less is known about the effect of gradual temperature changes within a range of 'comfortable' ambient temperatures. It is also unknown whether different temperature-gated TRP channels carry out redundant or complementary roles, or whether other mechanisms, besides

the activation of TRP channels, contribute to peripheral thermosensation. Most importantly, it is not yet well characterized how peripheral temperature signals mediated by TRP channels are integrated and evaluated at the level of the spinal cord and brain to evoke behavioural or physiological responses.

B) Integration of environmental information by the preoptic area and the hypothalamus

The anterior hypothalamus and the preoptic area (POA) are important areas for temperature regulation (Hensel 1973). The best-known thermosensory pathway is the spinothalamocortical pathway, which mediates conscious perception and discrimination of skin temperature (Nakamura & Morrison 2010). Temperature signals from the peripheral nervous system originating from dermal and mucosal thermoreceptors (Bratincsak & Palkovits 2004) reach the brain via the dorsal root ganglia, the spinal cord and the lateral parabrachial nucleus (LPB), which then projects to the POA (Caterina 2007; Nakamura & Morrison 2010). The POA itself contains thermosensitive neurons that respond to temperature changes of blood and brain tissue (Bratincsak & Palkovits 2004; Tattersall & Milsom 2009). In total about 30% of hypothalamic neurons are directly warm-sensitive and increase their firing rates when hypothalamic temperature increases. Cold and warm stimuli do not seem to activate the same neuron populations within the brain, suggesting different neural pathways (Kiyohara et al. 1995; Bratincsak & Palkovits 2004; Yoshida et al. 2005). Increased activity of hypothalamic warm-sensitive neurons activates heat loss mechanisms, followed by a decrease in body temperature, whereas stimulation of cold-sensitive neurons results in the opposite effects (Baarendse et al. 2007). For example, hypothalamic vasopressin (VP) neurons are intrinsically thermosensitive (Sharif-Naeini et al. 2008), leading to preventative water reabsorption in the kidney through the release of vasopressin under temperature stress. Based on immediate early gene studies, other areas of the hypothalamus, such as paraventricular, dorsomedial and ventromedial nuclei and the posterior and lateral hypothalamic areas have been implicated in the processing of information related to thermoregulation (Yoshida et al. 2005). However, even though several brain regions involved in thermosensation and -regulation have been identified in mammals, the precise cellular mechanisms that mediate neuronal thermosensitivity are not well known (Griffin et al. 1996).

The POA is thus an area that not only integrates input from ascending neural pathways carrying information derived from receptors in the periphery, such as the epidermis, but also the location of neurons sensitive to internal body temperature. The POA is therefore responsible for eliciting the thermoregulatory responses that are appropriate for both internal and environmental temperature assessments in homeotherms (Boulant & Dean 1986; Lipton & Clark 1986; Spray 1986; Hori et al. 1988; Boulant 2000). Neurons in the POA again provide a key input to neurons in the dorsomedial hypothalamus (DMH), which is an important site for the integration of

autonomic, endocrine and behavioral responses to environmental challenges (DiMicco & Zaretsky 2007). In rats, DMH neurons control a wide array of thermogenetic adjustments, including shivering and non-shivering thermoregulation, cutaneous vasoconstriction and endocrine changes involving the thyroid and adrenal gland with wide-ranging metabolic effects (DiMicco & Zaretsky 2007). The activity of both glands is under central control of neurons that regulate the release of the pituitary hormones ACTH and thyrotropin, respectively, and the common pathway controlling their release is through neurons in the paraventricular nucleus (PVN) (Bhatnagar & Dallman 1999; Herman et al. 2003; Engelmann et al. 2004).

It is important to note that in birds and mammals the same brain areas that receive information about the internal and external temperature also express various neuropeptides that regulate hormone systems that either control aspects of reproduction directly and/or control hormone systems that are candidate effector systems by which temperature might modulate reproduction (see below). For example, if we focus on birds, the cell bodies of the gonadotropin releasing hormone system (GnRH-I) that control the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) involved in gonadal maturation are present in the POA and the adjacent septum. Also cell bodies for the neuropeptides thyrotrophin-releasing hormone (TRH, regulating thyroid hormones) and vasoactive intestinal polypeptide (VIP, regulating prolactin) are present in the periventricular nucleus (PVN) of birds. Further studies are required to ascertain if thermosensitive cells in the POA interact directly with releasing hormone systems to modulate seasonal phenology.

C) Possible effector pathways that can affect seasonal timing

The anatomical setup of temperature sensation and integration is mainly known in the context of thermoregulation. Therefore our understanding of mechanisms that connect these neural processes to efferent pathways able to translate temperature cues into physiological processes in the context of seasonal timing is still comparably rudimentary.

Thyroid hormones are known to increase the metabolic rate and energy expenditure in general and are specifically permissive for heat generation in special tissues such as brown adipose tissue in some mammals (Freake & Oppenheimer 1995; Bianco et al. 2005; Laurberg et al. 2005). These metabolic effects appear to be restricted to mammals and represent a coordinated response to the challenge of maintaining body temperature constant (Freake & Oppenheimer 1995). However, they have also been suggested to be possible mediators of temperature effects on seasonal events (Wada 1993; Chastel et al. 2003). Thyroxine (T4) is secreted by the thyroid gland and peripherally converted into triiodothyronine (T3), which is the metabolically active form of thyroid hormones. Interestingly, birds, in contrast to mammals, lack a specific

T3-transporting protein in their blood, and thus the turnover rate of T3 is relatively high, which makes it a potentially more powerful agent in birds than in mammals (Chastel et al. 2003). Thyroid hormones, and especially T3, also play an important role in substrate metabolism and thermogenesis.

Different subspecies of the white-crowned sparrow (*Zonotrichia leucophrys gambelii*, *pugetensis* and *oriantha*) showed different sensitivities to temperature cues, with birds living at lower latitudes being more sensitive to temperature than birds breeding at higher latitudes (Maney et al. 1999). In these birds it has been shown that temperature effects are visible at the level of the gonads, but temperature also influences the concentrations of thyroid hormones, which were therefore suggested as possible mediators of temperature effects at the level of the gonads (Wingfield et al. 1996; Wingfield et al. 1997; Wingfield et al. 2003). In an experimental setup, Japanese quail (*Coturnix coturnix japonica*) only regressed their gonads outside the breeding season if in addition to short days also ambient temperatures were low (Wada et al. 1990; Wada 1993). Levels of T3 were more elevated in birds kept under cold temperature than in birds kept under warm temperature, which did not regress their gonads. In contrast, in birds kept under short photoperiods, but warm temperatures, T4 concentrations were markedly increased. Again, these results were interpreted such as effects of low temperature on gonadal regression might be relayed through thyroid activation, but the correlational nature of the data does not prove a direct effect of thyroid hormones on gonadal size. In house sparrows (*Passer domesticus*), birds that initiated breeding earlier in spring had both higher titres of plasma T3 and higher basal metabolic rates (BMR) during the pre-breeding period than birds that started to breed late (Chastel et al. 2003). This correlation indicates that thyroid hormones might influence timing of breeding indirectly, acting via its effect on BMR and thus indicating increased energy requirements prior to breeding. Thus, an indirect way in which temperatures could be integrated to influence the seasonal timing of life history decisions of homeotherms, especially birds, is by affecting energy balances via the hypothalamo-pituitary-thyroid pathway.

However, in birds, thyroid hormones are also involved in photoperiodic time measurement, which takes place in the mediobasal hypothalamus (MBH). Besides containing deep-brain photoreceptors directly measuring day length, the MBH also expresses circadian clock genes and thus may function as the avian 'photoperiodic clock' (Yamamura et al. 2006). In Japanese quail, the gene encoding the enzyme that catalyses the intracellular deiodination of T4 to T3 is induced by light (Yamamura et al. 2006), and administration of T3 mimics a photoperiodic response. The reaction to either light stimulation or presence of T3 is thus similar: Gonadotropin-releasing hormone-I (GnRH-I) neuron nerve terminals, which are enclosed by the end feet of glia cells in animals subjected to short day conditions, change morphologically such that nerve terminals are in close proximity to the basal lamina and secrete GnRH-I,

which eventually induces testicular growth. Experimental T3 infusion in the brain therefore induced testicular growth in a dose-dependent manner under short day conditions. For that reason, thyroid hormones seem to be essential for transitions among reproductive states over the breeding season in both mammals and birds (Yamamura et al. 2006), as well as being a vector of integration of both photoperiodic and temperature cues. GnRH in turn activates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary, which induce gonadal growth and eventually ovulation. However, experimental evidence suggests that higher temperatures do not necessarily lead to an elevation in LH levels (Silverin & Viebke 1994; Silverin et al. 2008; Schaper et al. 2012), of which a surge is essential at the end of the exponential growth phase of the follicle for inducing ovulation. Therefore the neuroendocrine mechanisms modulating gonadotropins in response to temperature cues are still unresolved.

Hypothalamic thyrotrophin-releasing hormone (TRH) is the neurohormone that ultimately induces the release of thyroid hormones from the thyroid gland via actions on thyroid stimulating hormone (TSH) that is synthesized and released by the anterior pituitary. In rats, cold exposure caused an increase in TRH concentrations in the PVN (Luza et al. 2003; Fiedler et al. 2006; Jara et al. 2010). Interestingly, TRH also serves as a neurotransmitter and thereby provides a sympathetic (i.e. non-neurohormonal) pathway between the brain and the ovary, and this neural connection thereby provides an additional possibility how temperature cues integrated in the hypothalamic PVN might directly impact on the ovary function and activity in mammals (Luza et al. 2003; Fiedler et al. 2006; Jara et al. 2010). Anatomical studies have localized TSH in the PVN of the avian brain, similar to the location in mammals (Jozsa et al. 1988) but studies on temperature effects on TSH or the possible modulation of ovarian function via the sympathetic nervous system have not been completed in birds.

Besides thyroid hormones, the pituitary hormone prolactin (PRL) has also been discussed as a mediator of temperature effects in avian reproduction. In three subspecies of white-crowned sparrows exposed to experimental temperature regimes, increases in prolactin concentrations accompanied an accelerated gonadal growth induced by higher temperatures. This led the authors to hypothesize that PRL mediates temperature cues, for example through a positive influence on the number of gonadotropin receptors at the level of the gonads (Maney et al. 1999). In golden hamsters (*Mesocricetus auratus*), injections of PRL indeed induced testis growth and the number of LH receptors in the testes (Bex et al. 1978). Yet, the observed differences in PRL concentrations may be either a cause or a result of gonadal development under different temperature regimes. Photostimulated turkey hens (*Meleagris gallopavo*) showed increased plasma PRL concentrations and sexually matured faster under higher temperatures. The increase in PRL associated with sexual maturity was faster in birds under warmer temperatures, however, PRL concentrations were rather

associated with a faster gonadal development than a result of higher temperatures *per se*, because caged individuals exposed to the same temperatures maintained low PRL titers and late onset of sexual maturity (El Halawani et al. 1984). Furthermore, chronic exposure to either high or low temperatures had no effect on the photoinduced rise in plasma PRL, whereas a temperature reversal from low to high temperatures induced a significantly greater PRL increase than a shift from high to low temperatures (Gahali et al. 2001). The physiological implications and the mechanisms underlying temperature effects mediated by PRL are thus far from clear. Elevated PRL titers are associated with incubation behaviour, gonadal regression and the onset of moult, which have also been shown to be temperature-dependent (Dawson & Sharp 1998; Dawson & Sharp 2010), but not in all studies (Dawson 2005a; Visser et al. 2011a; Schaper et al. 2012). The involvement of PRL in the onset of avian reproduction has not been unambiguously proven and mechanistic evidence on possible pathways is to date lacking.

Variation in temperature sensitivity

The seasonal timing of crucial events, such as the onset of reproduction or migration, is highly synchronized in both mammals and birds. However, the start date of these events shows considerable variation between distinct populations, for example due to latitudinal or altitudinal differences in the onset of spring. In addition, the mechanisms controlling seasonal timing exhibit a form of plasticity in that life-cycle stages can be adjusted to the conditions in a given year (for example, many birds start to breed and migrate earlier in a warm year (McCleery & Perrins 1998; Sokolov 2000; Dunn 2004) and individuals may differ in the degree of phenotypic plasticity in response to temperature cues (Nussey et al. 2005; Husby et al. 2010). Less is known about individual variation in the adjustment of phenological events in response to temperature changes in mammals as compared to birds. However, the individual variation in timing of a seasonal event in a given year can often be as large as the variation between years (Nager 1990). These individual differences within a given population could be partly explained by genetic differences in sensitivity to temperature cues (Visser et al. 2011a; Schaper et al. 2012). Unfortunately, evidence for local or individual differences in temperature sensitivity so far only exists in birds.

A) Population-level differences in temperature sensitivity

At a subspecies or population level, clear differences in the onset of reproductive development have been demonstrated in several bird species from observational studies and in some cases solidified by experimental approaches. These changes can be interpreted as differences in sensitivity to photoperiodic or temperature cues.

In great tits originating from different latitudes, the onset of the rapid testicular growth phase differed between individuals from different populations in a common garden experiment. Male birds from Northern Italy developed their gonads early, followed by birds from Southern Sweden and Northern Norway (Silverin et al. 1993) under the same increasing photoperiod. Additionally, the initial growth rate and luteinizing hormone (LH) secretion was affected by ambient temperature (4 vs. 20°C) in the Italian population only (Silverin et al. 2008).

Differences in the responsiveness to environmental cues were also observed in three subspecies of white-crowned sparrows in Western Washington State: different subspecies show different sensitivities to temperature cues, with birds breeding at lower altitudes being more sensitive to temperature cues than birds breeding at higher altitudes (Wingfield et al. 1996; Wingfield et al. 1997; Wingfield et al. 2003). Variation in the extent in which supplementary cues, such as temperature, were taken into account correspond with the predictability of the breeding season in different habitats (Wingfield et al. 1992; Wingfield et al. 1993). However, testis development was not affected by direct temperature cues (constant temperatures of 5, 20 and 30°C) in any of the tested populations (Wingfield et al. 1996; Wingfield et al. 1997; Wingfield et al. 2003).

A coastal and a montane population of song sparrows (*Melospiza melodia morphna*) in Western Washington were compared with respect to their timing of gonadal growth in response to temperature cues. In nature, timing differs between these two populations by as much as two months, correlated with variation in ambient temperature at each site. When exposed to a constant experimental temperature (15°C), testes grew at the same rate (Perfito et al. 2004). When birds were held on temperatures typical for each site (montane or coastal temperature profiles), only birds from the montane population adjusted the timing of testis development in response to temperature cues (Perfito et al. 2005).

B) Individual variation within populations

There is little experimental evidence for individual variation in temperature responsiveness related to reproductive timing. In controlled experiments, breeding pairs from the same population of genetically related individual great tits were exposed to a natural increase in photoperiod, but various thermal environments in climate-controlled aviaries. These experiments indicated that sisters from the same family showed a similar reaction to temperature cues in terms of their onset and termination of egg laying (Visser et al. 2011a; Schaper et al. 2012).

Temperature perception in the epidermis can possibly also be influenced by fur colour in mammals. European ground squirrels (*Spermophilus citellus*) retreat to the temperate climate of the burrow system to avoid high surface temperatures on sunny

days and midday heat decreases midday activity. A manipulation of head coat colour and hence ambient temperature perception showed that significantly fewer dark-headed squirrels were visible on the surface in midday on sunny days (Vaczi et al. 2006). It would be interesting to further investigate if darker morphs not only change their behaviour in response to temperature, but possibly also advance temperature-dependent seasonal activities.

C) Ontogenetic effects on temperature adaptation

The ontogeny of important neural structures involved in avian thermosensation has been described in chicken and ducklings. In these precocial birds, maturation of hypothalamic temperature sensitivity takes place during the late prenatal and early postnatal period (Baarendse et al. 2007). Muscovy ducks (C*airina moschata*) are characterized by a high neuronal cold sensitivity and a low neuronal warm-sensitivity during the time-period comprised between late incubation and day 5 post-hatching. Later on, during the homeothermic phase, the neuronal cold sensitivity decreases whereas the proportion of warm sensitive neurons significantly increases (Tzschentke & Basta 2000). There is further evidence for plasticity in neuronal thermosensitivity at the prenatal and early postnatal phase, measured by the firing rate of neurons in the preoptical area of the anterior hypothalamus during sinusoidal temperature changes (Tzschentke & Basta 2000; Tzschentke & Basta 2002). Incubation temperatures (between 35 and 38.5°C) had a clear influence on postnatal thermosensitivity: exposure to cooler incubation temperature resulted in elevated warm sensitivity later in life through an increased proportion of warmth-sensitive neurons and a reduced proportion of cold-sensitive neurons, whereas exposure to warmer incubation temperature induced the opposite effect (Tzschentke & Basta 2002). These plastic changes can affect thermoregulation and performance in later life (Baarendse et al. 2007) and possibly also seasonal timing in response to temperature cues. Shortly after hatching, peripheral and deep-body thermoreceptors are also already functional in altricial species, in which thermoregulation is still immature at hatch (Baarendse et al. 2007).

D) Seasonal variation in thermosensitivity

How the neuronal receptiveness for temperature cues is changing seasonally in an individual is not sufficiently understood in birds. In mammals, estrogens have distinct effects on thermoregulation (Dacks & Rance 2010) and could therefore also be involved in the regulation of thermosensitivity in relation to reproductive state. Three possible sites of integration between the reproductive and thermoregulatory axes have been described in female ovariectomized rats. In the median preoptic nucleus (MnPO), the arcuate nucleus and the anteroventral periventricular nucleus (AVPV), a treatment with estradiol-17β (E2) modulated the electric activity of warm-

sensitive neurons (Dacks et al. 2011). However, it remains unknown if, or how, birds and mammals might use these receptors and brain regions to detect and integrate seasonal temperature information over long time periods or with the sensitivity required to detect annual differences in average temperatures of only a few degrees (Williams 2012).

The roadmap to the future

A complete understanding of thermoreception includes the identification of relevant temperature cues, the measurement of physiological and behavioural reactions in response to these cues, and the investigation of neural and endocrine events evoking these responses. It is obvious that to reach these goals, the fields of avian and mammalian physiology and seasonal timing can greatly benefit from a more thorough cooperation. While some aspects, such as the means of thermoreception, are most likely applicable to all homeotherms, the timing of life-cycle stages such as reproduction and moult in birds and mammals are obviously based on different strategies and thus vary between these taxa. To better integrate our understanding of similarities and functional differences between these two groups, we advocate several approaches.

Most work on the correlational and causal relationship between seasonal timing and temperature cues is done in birds, but the actual physiological mechanisms mediating temperature information were investigated in mammals, especially rats and mice. The few studies in birds mostly make use of chicken and pigeons. In the context of seasonal timing, more non-model (and non-domesticated) species that show a high degree of seasonality should be used in future experiments. In addition, we should take advantage of the genetic variation among natural populations of the same species that evolved over diverse seasonal climatic gradients (Bradshaw & Holzapfel 2010).

To investigate seasonal changes in thermosensitivity, one needs to test birds and mammals under seasonal conditions to find out more about variation in the underlying physiological mechanisms over time, such as changes in receptor densities. In this context the role that the internal state, such as the stage of sexual maturation, plays in influencing seasonal changes need to be further clarified. In addition, the interplay between circannual rhythms, photoperiodism and temperature sensitivity needs to be explored.

So far, knowledge about thermoreception involves the identification of components that are part of the receptor or effector systems in relation to thermoregulation or the handling of pain, thus focusing on temperature ranges that are extreme compared to the annual differences in average temperatures of only a few degrees. Besides using more moderate temperatures and possibly testing the activity of sensory systems in different times of the season, also reactions to (more subtle) temperature changes, in contrast to constant temperatures, should be investigated in more detail.

From a different angle, variation in temperature sensitivity on a population level should be assessed using a quantitative genetic approach. This requires accurate phenotypic, pedigree and genotypic data from a large number of individuals, but is feasible as several long-term studies exist on both birds and mammals (Clutton-Brock & Sheldon 2010) that facilitate the identification of quantitative trait loci (QTLs).

To conclude, we know that temperature affects seasonal timing strongly in birds, but temperature effects on seasonal activities such as parturition in mammals are less pronounced. In contrast, the neuronal and endocrine mechanisms underlying the perception, transduction and integration of temperature stimuli are mainly derived from studies on mammals and might show substantial differences in birds. The inclusion of more seasonal avian species in physiological studies could be better suitable for the identification of pathways that can accommodate moderate temperature cues from the environment and thus influence seasonal timing in homeotherms. In this context, thyroid hormones are promising candidates, as they are directly involved in thermoregulation and thus associated with different pathways integrating temperature cues anyways, but also because different possible thyroid-mediated connections between thermoreception at the level of the brain and seasonal events, such as maturation of the gonads, have been identified. Further studies should be performed on both the influence of temperature cues on reproductive timing and the integration between cues and reproductive physiology. Complementary, research that aims at the identification of candidate genes underlying the variation in thermosensitivity is crucial to understand the causal relationship between temperature cues and seasonal timing.

Chapter 9

Luc te Marvelde, Sonja V. Schaper and Marcel E. Visser

A single long day triggers follicle growth in captive female great tits (*Parus major*) in winter but does not affect laying dates in the wild in spring

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Abstract

In many forest passerine bird species, rapid climate warming has led to a phenological mismatch between the period of maximum food requirements (large nestlings) and the period of maximum food availability (seasonal caterpillar biomass peak) due to an insufficient advancement of the birds' laying dates. The initiation of laying is preceded by the development of the gonads, which in birds are regressed outside the breeding season. Increasing day length in late winter and early spring triggers a cascade of hormones which induces gonadal development. Since day length is not altered by climate change, one potential restriction to advancing laying date is the seasonal timing of gonadal development. To assess the importance of gonadal growth for timing of reproduction we experimentally manipulated the timing of gonadal development. We show that the growth of the largest follicle of captive female great tits (*Parus major*) increased after being exposed to just a single long day in winter (20 hours of light followed by 4 hours darkness). We then photostimulated wild female great tits from two study areas in a field experiment in spring for a single day and determined their laying date. These populations differed in the availability of food allowing us to test if food availability in combination with photostimulation affected egg laying dates. Despite an expected difference in the onset of gonadal growth, laying dates of photostimulated females did not differ from control females in both populations. These results suggest that wild great tits are not restricted in the advancement of their laying date by limited gonadal development.

Introduction

Breeding success largely depends on the timing of breeding relative to the timing of maximum food availability (van Noordwijk et al. 1995; Verboven & Visser 1998; Visser & Verboven 1999). Many forest passerine bird species in temperate regions feed their young with caterpillars, which occur only in a short period of time during spring (caterpillar biomass peak). Due to increased spring temperatures, the window in which food availability is high has shifted forward in time over the last 25 years (Visser et al. 2006). As a result, the optimal timing for breeding advanced, but many species, like the great tit, have not adjusted their timing sufficiently, causing them to breed too late (Visser et al. 1998; Crick & Sparks 1999; Visser et al. 2006).

The reproductive system of most seasonally breeding birds, including great tits, shows a clear seasonal pattern (Murton & Westwood 1977; Silverin 1994). Gonads are regressed during winter, grow slowly during late winter and grow rapidly in spring until they are fully developed. Gonads are regressed again after the breeding season. The rapid growth in spring is affected by increasing day length, which causes the release of gonadotropins (Murton & Westwood 1977; Dawson et al. 2001; Sharp 2005). Although it has been shown that temperature can affect the speed at which gonads develop in great tits breeding in Southern latitudes (Silverin et al. 2008), the

speed of gonadal development in great tits breeding in more Northern latitudes is not accelerated by increasing spring temperatures (The Netherlands (Schaper et al. 2012), Scandinavia (Silverin et al. 2008)). In these latitudes, gonadal growth is driven by photoperiod (Silverin et al. 2008). Increasing spring temperatures due to global warming will therefore not advance the birds' readiness to reproduce. As climate change does not affect the seasonal change in photoperiod, a possible reason as to why great tits are not advancing their laying date adequately is that gonads are not fully developed early enough to allow early egg laying.

To test whether gonadal development is hampering early egg laying, gonadal development needs to be experimentally advanced. This could be done by manipulating the photoperiod a bird experiences, as shown in an experiment where blue tits (*Cyanistes caeruleus*) in captivity (with *ad libitum* food) could be tricked into laying their eggs in winter (January) by exposing them to long days from December onwards (Lambrechts & Perret 2000). Under a natural photoperiod, egg laying in January is not possible as the reproductive system will not be fully developed at that time.

Although photoperiod is easily manipulated in captive birds, photostimulating birds in the field has many practical problems, such as fitting a large number of nest boxes with a light, batteries and a timer. More importantly, birds do not always sleep in a particular nest box in the period before egg laying and thus it is difficult to determine which bird is photostimulated and to what extent. Taking wild birds into captivity for photostimulation treatment for long periods can cause problems as breeding vacancies resulting from the removal of territorial birds are filled within a few days by unpaired birds, which may lead to fights after release of the original territory holder or its female (pers. comm. P. de Goede, NIOO-KNAW).

Previous experiments have shown that the exposure to a single long day can affect levels of hormones involved in reproduction. Nicholls et al. (1983) kept Japanese quail under 8L:16D (8h light, 16 h darkness) and gave them a single long day of 20L:4D resulting in an increase of luteinizing hormone (LH) and follicle stimulating hormone (FSH) within four hours from the end of the long day. These birds were kept in constant darkness thereafter and their LH and FSH levels decreased slowly over the next 8 to 10 days. Creighton and Follett (1987), who performed a similar experiment, kept Japanese quails under short day lengths after just one long day and report that LH remained elevated for three days after photostimulation. Follett et al.(1975) have shown that LH levels of white-crowned sparrows (*Zonotrichia leucophrys gambelii*) transferred from short (8L:16D) to long days (20L:4D) increased six-fold in five days, with the largest increase after the first day (three-fold increase). Saab et al. (2010) have shown that a single long day increased gonadotropin-releasing hormone and LH concentrations in white-throated sparrows (*Zonotrichia albicollis*). A single long day also affects gonadal growth of both male and female song sparrows (*Melospiza melodia*). Wingfield (1993) showed that after one single long day, females' gonads

grew for up to 60 days, even though the changes in LH and FSH were only present for a few days. In all of the experiments mentioned above food was available *ad libitum*. If a single long day would affect gonadal growth in wild female great tits, it would allow us to test the hypothesis that laying dates are restricted by photo-induced gonadal growth.

Outside the breeding season gonads are regressed, implying that there are costs connected with having and/or maintaining fully developed gonads. These costs can be present in terms of increased risk of predation due to lower aerial manoeuvrability and take-off ability (Hedenstrom 1992; Witter et al. 1994; Metcalfe & Ure 1995), but might also involve energetic maintenance costs. Thus, it is likely that advancing gonadal growth in spring also comes with a cost. If energetic costs restrict early gonadal development, only birds in habitats with high food availability might be growing their gonads as a reaction to the exposure to a single long day.

The aim of this study was to determine 1) if a single long day induces gonadal growth in captive female great tits and 2) if a single long day in spring affects laying dates in two field populations which differ in the availability of supplementary food in the period before and during egg laying. If gonadal growth restricts early egg laying, we expect photostimulated birds to lay earlier compared to control birds in both study areas. If gonadal growth is restricted by a combined effect of photoperiod and food availability, we expect only those birds in the population with available supplementary food during the pre-laying period to advance egg laying.

Materials and Methods

Ethics Statement

The experiments reported here comply with the current law in The Netherlands and were carried out under licenses of the Animal Ethics Committee of the KNAW (Royal Netherlands Academy of Arts and Sciences, protocol CTE.08.10 & CTE 09.01).

Study areas

This study was carried out in two study areas, Hoge Veluwe and Oosterhout (the Netherlands), about 50 km apart. Strong natural selection for early laying females exists in both populations (Visser et al. 1998 and unpubl. data MEV). The study sites were chosen for this experiment because food availability in the period before egg laying differs between them. National Park 'De Hoge Veluwe' (52° 02' 07" N 5° 51' 32" E) is a mixed forest on poor sandy soils, while Oosterhout (51° 52' 22" N 5° 50' 22" E) is a rich deciduous forest on rich river clay. Besides finding food in the rich undergrowth, females of the Oosterhout population regularly fly to the nearby village (maximum

distance 1 km) to feed on the abundant supplemented food (observed during radio tracking, unpublished data LtM & MEV). This food was available until most birds laid their eggs and consisted mainly of fat and peanuts. Over the last 25 years, natural selection favoured early breeding in 21 of the past 25 years in Oosterhout and in 23 out of 25 years in the Hoge Veluwe (Visser et al. 2006, unpubl. data MEV).

Aviary experiment

To test if one (or two) long days initiate gonadal growth, we caught 15 wild female great tits at the end of November 2008 around the Netherlands Institute of Ecology, Heteren (The Netherlands; 51° 57' 20"N - 5° 44' 34"E). All females were housed in one large outdoor aviary under natural light and temperature conditions and *ad libitum* food and water. Six days before the light treatment (5th December 2008), length of the largest ovarian follicle of all females was measured during laparotomy without knowledge of the treatment each female would be assigned to. To measure follicle development, a small incision was made between the last two ribs on the left side. By parting the ribs slightly, the length of the largest follicle was measured to the nearest 0.1 mm with an ocular scale. When the length of the largest follicle was too small to be measured (significantly smaller than 0.1 mm) we reported a length of 0.05 mm (n=3 in December). All laparotomies were carried out by SVS under light Isoflurane anaesthesia. Follicle volume was calculated as $V=4/3\pi a^3$ where a is ½ the length of the follicle.

For the first photostimulation treatment (11th December 2008), all females were moved indoors after sunset into individual cages in two separate rooms (see Fig. 9.1 for a schematic overview of the treatments). Ten females in one room were exposed to light for 20 hours (7am – 3am), then darkness for four hours, after which the lights were turned on again at 7am the following day. Five control females in the second room were kept under the natural light regime (light from 8:40am to 4:30pm). All females were returned to the outdoor aviary the day after the photostimulation treatment. Seven days later (18th December 2008), five of the 10 photostimulated females were photostimulated again using the same protocol, while the other five photostimulated females and five control birds were also kept indoors but under natural photoperiod. Thus, the aviary experiment consisted of three treatment groups, each containing five birds: 1) natural short photoperiod 2) one long day and 3) two long days with an interval of one week in between. One month after the first photostimulation (15th January 2009) the length of the largest follicle was measured again for each female. One week after the last laparotomy all 15 females were released into the wild at the catching location (Fig. 9.1).

Figure 9.1: Schematic overview of the photostimulation treatment for both the aviary and the field experiment. For the experiment in captivity (December 2008), females were moved from the outdoor aviary to our indoor facilities after sunset, where a part of the females were kept under natural day length and a part were kept in a different room under a long day length (20L:4D). Females were moved to their outdoor aviary in the morning of day three. For the field experiment (March 2009 and 2010), females were brought from the field into out indoor facilities for a similar treatment. On the morning of day three, all females were released in the field at the location where they were caught. Grey bars resemble darkness while open bars represent light periods; numbers in the bar are the hours of the day.

Field experiment

We carried out a field experiment to test if laying dates in the wild are restricted by gonadal development of the females. In the Oosterhout (~150 nest boxes) and Hoge Veluwe study area (~440 nest boxes) all nest boxes were checked at night for the presence of female great tits (Oosterhout 2009: 25th February; 2010: 1st March; Hoge Veluwe 2009: 3rd March). All females were banded with a uniquely numbered aluminium ring as well as a unique colour band combination. Of the 103 females encountered, we took 69 females into temporary captivity (Oosterhout: 2009 n=20, 2010 n=20, Hoge Veluwe: 2009 n=29; Table 9.1). Thus, 34 females were not taken into captivity; these control females were put back into the nest box after being ringed and weighed with a pesola spring scale (Table 9.1). The females taken into captivity were housed indoor in individual cages with *ad libitum* food and water.

Half of the females were kept indoors under a natural light regime (light from 7:30 am to 6:15 pm), while the other half were photostimulated (light from 7 am to 3 am; Fig. 9.1) and thus experienced an increase in day length of 8 hours and 45 minutes. Thus, we created three experimental groups in this field experiment: 1) photostimulated females in captivity 2) females which experienced natural day length in captivity and 3) females which experienced natural day length in the field. We chose not to measure gonadal size of the females in the field after release since disturbance in the period just before egg laying could probably affect the timing of laying. All females were released at the field site of capture after the day after the treatment (day 3, Fig. 9.1).

Nest boxes at both study sites were checked once a week from the beginning of April onwards to monitor nest building. Once the bottom of the box was covered with nest material, nests were checked daily to determine the exact laying date (date the first egg was laid). During incubation, females were identified by their unique colour code combination by lifting them slightly with a pen to expose the colour rings. Laying dates of three females were excluded since they were likely to be replacement clutches (>30 days after the first egg laying dates in that year of that population) of which we had missed the first clutch (removed laying dates are: Oosterhout 2009: 40th April; Oosterhout 2010: 34th April and 61st April). Results of the analysis did not change after including these data points.

Statistical analyses

We used a One-sample Wilcoxon signed rank test to test if follicles grew for the photostimulated females in the aviary experiment and a Two-sample Wilcoxon signed rank test to test if gonadal growth was different for females which were photostimulated either once or twice. To test for differences in laying dates in the field experiment we used ANOVAs. Because the experiment was done in Oosterhout in 2009 and 2010 and in the Hoge Veluwe population only in 2009, we divided the

analyses of the field experiment in two parts; comparison between study areas in 2009 and comparison between years for the Oosterhout population. All statistics were done using R 2.9.2 (R Development Core Team 2009).

Results

Aviary experiment

Gonadal growth of female great tits in captivity could only be determined for 9 out of the 15 females due to technical difficulties (two control females, three females photostimulated once and four individuals photostimulated twice in December were measured). Females that were photostimulated once or twice (20L:4D) showed gonadal growth one month after the treatment (One-sample Wilcoxon signed rank test: V=28, P=0.016), whereas both females of the control group did not show gonadal growth. Gonadal growth of females which were photostimulated once did not differ from females that were photostimulated twice (Two-sample Wilcoxon signed rank test: W=5, P=0.86; see Fig. 9.2).

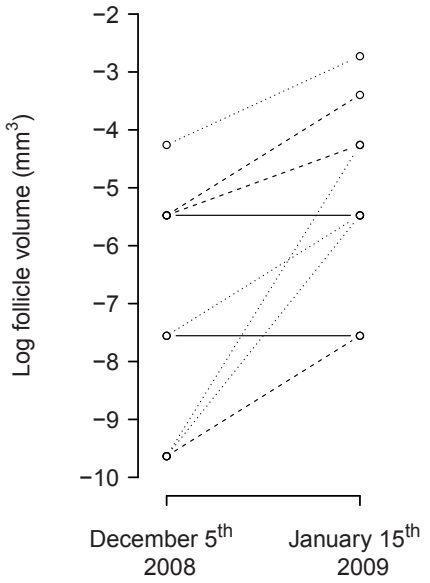

Figure 9.2: Gonadal development of the three experimental groups in the aviary experiment before and one month after the photostimulation. All birds were kept in an outdoor aviary and only moved indoor for the photostimulation treatment. Of the females of which we successfully measured the largest follicle in December and January, 2 birds were kept under natural light conditions (solid lines), 3 individuals were given one long day (dashed lines) and 4 individuals were given two long days (seven days apart; dotted lines). Follicle volume was square root transformed for graphical purposes.

Field experiment

In total, 63 out of 103 females in the field experiment started egg laying in our study area (Table 9.1). Females started laying on average (± SE) on April 18.0 (± 1.17) , 14.3 (± 0.69) and 16.7 (± 1.0) in the Hoge Veluwe 2009, Oosterhout 2009 and 2010, respectively. Laying dates of first eggs did not differ between photostimulated and control female great tits in either of the two years and in either of the two study areas (P>0.22 for all comparisons; Table 9.2; Fig. 9.3).

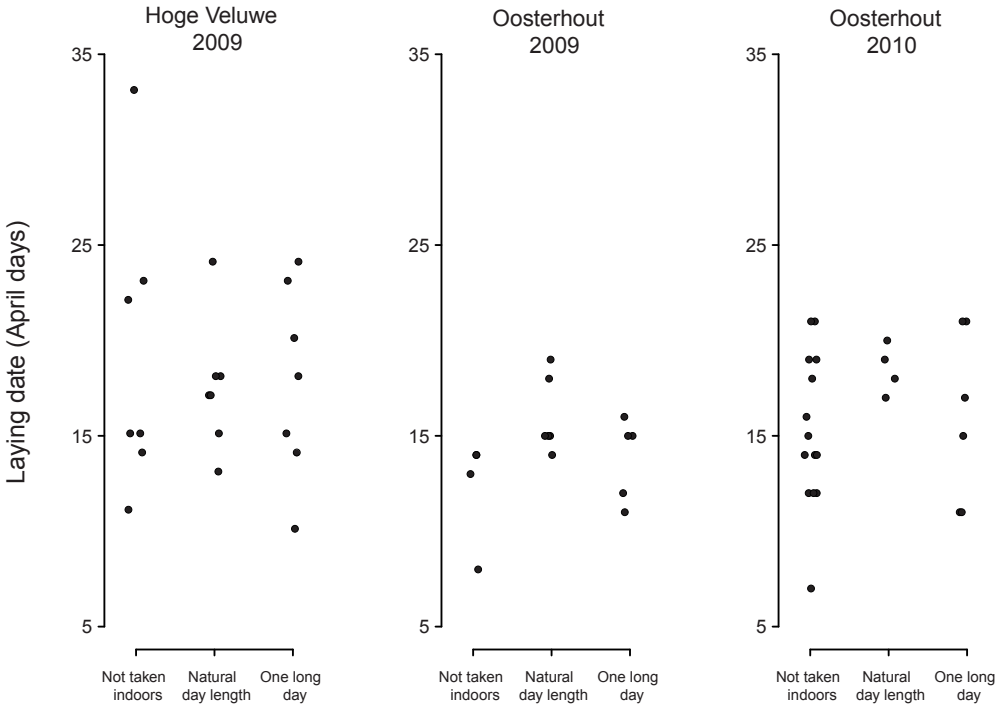

Figure 9.3: Laying dates (date when the first egg of a clutch was laid) of females in the field experiment. One third of the females encountered during a night check of all nest boxes were not taken indoors, 1/3 were taken into captivity and kept under natural light regime and 1/3 were taken into captivity and kept under a long light regime (according to Fig. 9.1). Laying dates in April days, where 1 = 1st April. Note that random jitter is applied (on the X-axis only) to separate overlapping data points.

Table 9.1: Sample sizes of the field experiment to study the effect of photostimulation on laying dates in wild great tits (*Parus major*). We created three experimental groups: 1) photostimulated females, 2) females which experienced natural day length in captivity and 3) females which experienced natural day length in the field. Females from group 1 and 2 were kept indoors for one day and two nights after which they were released in the field at the location where they were caught.

		Treatment	Sample size	Birds with laying date	% with laying date
Oosterhout	2009	Indoor - one long day	10	6	59%
		Indoor - natural day length	10	6	
		Not taken indoors	7	4	
	2010	Indoor - one long day	10	7	72%
		Indoor - natural day length	10	5	
		Not taken indoors	16	14	
Hoge Veluwe	2009	Indoor - one long day	15	7	53%
		Indoor - natural day length	14	7	
		Not taken indoors	11	7	
Total indoor			69	38	55%
Total not taken indoor			34	25	73%
Totals			103	63	

Table 9.2: Results of the statistical analyses (ANOVAs) of the effect of photostimulation on first egg laying dates (date at which the first egg of a clutch is laid) of wild great tits in the field experiment. The experiment had three treatments: indoor photostimulated; indoor natural light regime; not taken indoors. The experiment was done in two locations in 2009 and in one location in 2010. We divided the analyses in two; 1) only data from the Oosterhout population (2009 and 2010) and 2) only data from 2009, where the experiment was done in the Oosterhout and Hoge Veluwe population.

Subset	Explanatory variable	df	Error df	Sum of squares	Mean squares	F	P
Oosterhout	Treatment*year	2	33	1.05	0.53	0.05	0.96
Oosterhout	Treatment	2	36	38.39	19.19	1.59	0.22
2009	Treatment*location	2	30	40.35	20.18	0.96	0.39
2009	Treatment	2	33	3.02	1.51	0.06	0.94

Discussion

This study aimed to test the hypothesis that timing of egg laying is restricted by the timing of gonadal development, which is under photoperiodic control. We showed that, even in winter (average daily temperature in the outdoor aviary in December 2008=1.8°C, January 2009=0.1°C; February=2.7°C), gonadal growth was initiated after exposing captive female great tits to 'a single long day' (20L:4D). In a field experiment in spring, however, free living female great tits which were given a single long day did not advance egg laying, either in the study area with or without good food conditions and the availability of supplementary food in the period before egg laying. Although we did not measure gonadal development prior to egg laying in the field study and can therefore not confirm that our 'single long day' treatment also worked in spring, these results suggest that the seasonal timing of gonadal growth does not play a major role in restricting great tits from advancing their laying date.

Follicles of female great tits which were exposed to a single long day in winter started growing. As gonads are in a regressed state outside the breeding season and only start growing very slowly in early winter, follicles were still small in December (maximum 0.3 mm long). It is therefore difficult to measure their size: in 3 out of 15 cases, the follicle size in December was too small to be measured precisely. Follicle sizes in January ranged from 0.1 mm to 0.5 mm, which is still small compared to the accuracy with which we can measure them (0.1 mm). Although measuring follicles in winter is difficult, we have confidence in our measurements.

Both populations have a long history of selection for early laying: reproductive success, measured as the number of fledged offspring that recruited in the breeding population in the next year, was higher for early breeding females for 21 out of 25 years in Oosterhout and for 23 out of 25 years in the Hoge Veluwe (Visser et al. 2006, unpubl. data MEV). If gonadal growth would restrict egg laying, this would most likely occur in years with high spring temperatures since warm springs lead to early egg laying. Temperature in the period 16th March until 20th April correlates well with laying dates (Visser et al. 2006). In 2010, mean temperature in this period was high and indeed, the first pair that started egg laying in the Oosterhout study population was the earliest recorded laying date in the last 55 years in this study area. Although laying dates are early in years with warm springs, gonadal growth is not affected by temperature in the period before egg laying (Schaper et al. 2012). Therefore we would expect an effect of photostimulation (especially in 2010) if gonadal growth was restricting egg laying dates.

Although follicle volumes increased after one day of photostimulation in captivity during winter, photostimulation in spring did not affect laying dates in the field. There are a number of potential explanations.

We did not measure gonadal growth in a subset of the photostimulated animals in March. It is therefore possible that the photostimulation in March did not result in gonadal growth, causing the lack of effect in laying dates. To our knowledge, no studies have focused on the seasonal variation in the strength of the response in hormonal change or gonadal growth to a single long day. However, Silverin (1994) caught male great tits during different months of the year and exposed (a part of) them to a 20L:4D light regime for 100 days. Testis growth of male great tits caught in December was less than one millimetre after 10 days of photostimulation, whereas male great tits caught in March grew their tested on average just over 2 mm. Thus, at both dates photostimulation led to a reaction that was adequate for that time and developmental stage and it is therefore likely that follicle growth of the female great tits in our field experiment was stimulated by our experimental treatment in March.

Since the natural day length is shorter in winter than in spring, the photostimulation in winter was a relatively stronger stimulation compared to the photostimulation in spring. Silverin (1994) measured testis growth of male great tits exposed to two different light regimes (14L:10D and 20L:4D) and showed that the gonadal maturation was faster in the 20L:4D group compared to the 14L:10D group.

In our experiment in spring, the increase in day length was still 9 hours and 15 minutes for the photostimulated group, which is more than the increase in day length from 8L:16D to 14L:10D in the experiment from Silverin (1994) which resulted in clear effects on hormones and gonadal development. We therefore believe the photostimulation treatment in spring is strong enough to evoke a response in gonadal growth. However, no studies exist showing that the length of a single long day affects hormones or gonadal growth differently.

Another possible explanation why photostimulated females did not advance egg laying is that, besides the primary predictive cue of photoperiod, supplementary cues are used to time egg laying (Farner & Wingfield 1980), for example the increase in spring temperature (Visser et al. 2011a; Schaper et al. 2012). One of the supplementary cues might be food availability. During the aviary experiment, as well as in most other experimental studies (e.g. Visser et al. 2011a; Schaper et al. 2012), food and water were given *ad libitum* while the females in the field encountered all kinds of stressors (predation risk, lower food availability, inter-species interactions etc.). In addition, eggs are laid in cold weather conditions under which foraging efficiency is low (Avery & Krebs 1984) and energetic costs are high (Stevenson & Bryant 2000; te Marvelde et al. 2012). Therefore, adverse food conditions can restrict growth of gonads (either acting as a cue or as an energetic constraint). Perfito et al. (2008) showed that male zebra finches (*Taeniopygia guttata guttata*) under long photoperiod but with food restrictions did not develop their testes, similar to those under short day lengths, while birds under long photoperiod with *ad libitum* food did. Zebra finches, however, are opportunistic breeders that have evolved to use food availability as a cue, since they live in areas where food availability is unpredictable and does not follow a seasonal

pattern (Zann et al. 1995). Testis size of male European starlings kept in aviaries was not affected by a food restriction (Dawson 1986), however, as the birds were able to maintain body weight during the treatment they were possibly not restricted enough. Also, in most physiological studies like these, males are used, while it is the females that determine the timing of reproduction (Caro 2012). It is likely that the female great tits in our field experiment were food restricted. If this would be the case, we would expect females in Oosterhout (which had a richer food supply) to advance egg laying compared to the control females, but not the Hoge Veluwe females. However, photostimulated females from the Oosterhout population also did not advance laying compared to the control groups in neither of two years. Therefore, we hypothesize that other supplementary cues, like temperature, or perhaps the availability of insects, prevented photostimulated females from laying early relative to the phenology of their environment.

While egg laying can not start before males and females have fully developed reproductive organs, we only photostimulated female great tits in the field experiment. Field observations showed that the male reproductive system is functioning well in advance of that of the female's (Farner & Wingfield 1980). Gonadal measurements of great tit breeding pairs in captivity confirmed this by showing that males have mature gonads sometimes weeks earlier compared to the exponential growth phase of female follicles (Visser et al. 2011a; Schaper et al. 2012). Therefore, laying dates in wild birds are not likely to be restricted by the development of the male reproductive system.

It is important to know which factors hamper the lack of a shift in laying dates because these can have different implications on how to adapt to future climate change. If a shift in laying date is hampered by gonadal development (which our result suggest not to be the case), birds have to adjust their rules in which day length is used as a cue. Since climate change is not affecting day lengths, gonadal development is likely to restrict a shift in laying dates in the future. At the moment there seems to be other reasons why the shift in the advancement of laying date lags behind this shift in the phenology of the food, leading to an increasing phenological mismatch between food availability and food requirements over the last decades. Future temperature increase will further this mismatch. A better understanding of the causes and consequences of the (in)ability of birds to adapt their timing of reproduction to restore the synchrony with their prey is important and will provide insights into the effects of future climate change on population viability (Visser 2008).

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Chapter 10

Sonja V. Schaper, Carolina Rueda, Peter J. Sharp, Alistair Dawson and Marcel E. Visser

Spring phenology does not affect timing of reproduction in the great tit (*Parus major*)

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Abstract

Many seasonal breeders adjust the timing of reproduction in response to year-to-year variations in supplementary environmental cues, amongst which ambient temperature is thought to be most influential. However, it is possible that for species such as the great tit (*Parus major* L.), phenological cues from sprouting vegetation and the consequent abundance of invertebrate prey, although dependent on temperature, may provide supplementary environmental cues *per se*. This hypothesis was investigated in breeding pairs of great tits kept in outdoor aviaries. In spring, experimental pairs were provided with access to leafing birch branches and caterpillars as a visual food cue, while control pairs were provided with non-leafing branches. Observations were made on the onset of laying and on concentrations of plasma luteinizing hormone (LH) at regular intervals to monitor changes in reproductive function. The onset of egg laying was not advanced by the presence of leafing branches and caterpillars. LH concentrations increased during the course of the study, but phenological cues did not affect plasma LH levels in females and males. Early spring vegetation, such as the leafing of birch branches, and the appearance of caterpillar prey do not appear to play a significant role in fine-tuning the onset of egg laying in great tits.

Introduction

Birds adapt their life histories to fluctuating environmental conditions, with energy-demanding activities, such as reproduction and moult, occurring at a time of year that offers sufficient energetic resources. Matching the rearing of nestlings with the seasonal food peak has large fitness consequences (Perrins 1965; van Noordwijk et al. 1995; Thomas et al. 2001; Sheldon et al. 2003; Charmantier et al. 2008), and there is strong selection pressure on mechanisms that enable females to predict future food availability from proximate environmental cues (Visser & Lambrechts 1999; Visser et al. 2010a).

In non-equatorial birds, lengthening photoperiod in spring provides the principal cue for the timing of seasonal breeding (Farner 1985; Follett et al. 1985; Silverin et al. 1993; Dawson et al. 2001; Sharp 2005), but supplementary cues derived from rainfall, ambient temperature and phenology are available to increase the precision of a timing decision (Wingfield et al. 1992; Meijer et al. 1999; Hau et al. 2004; Perfito et al. 2005; Salvante et al. 2007; Dawson 2008; Small et al. 2008; Visser et al. 2009; Schaper et al. 2012, but see Visser et al. 2011a). The aims of this study were, firstly, to re-evaluate evidence for the involvement of phenological cues in the onset of egg laying in opportunistic and strictly seasonally breeding birds and, secondly, to assess experimentally whether there is a causal relationship between phenological cues provided by leafing birch and caterpillars and the onset of reproduction in a seasonal breeder, the great tit (*Parus major* L.).

Opportunistic breeding birds live in environments in which the distribution of their food supply fluctuates erratically, and thus they require great sensitivity to environmental cues predictive of increased food supply to stimulate reproductive activity (Hahn 1998). Most studies have been carried out on birds living in arid regions with unpredictable rainfall (Table 10.1), and because of its importance for primary productivity, rainfall is still the climatic parameter most frequently analysed with regard to the onset of breeding (Barrientos et al. 2007). Rainfall stimulates the growth of vegetation, resulting in the production of leaves, flowers and seeds, and these not only provide plant and associated invertebrate food to feed nestlings but also may act as phenological cues for the initiation of breeding (Hahn et al. 2008). For example, in the granivorous zebra finch (*Taeniopygia guttata*) in central Australia, hatching coincides with the availability of grass seeds to feed nestlings after the onset of rain, with heavier rainfall resulting in longer breeding episodes and repeated rainfall stimulating repeated breeding (Zann et al. 1995). A similar phenomenon is observed in Darwin's ground finches (*Geospiza* spp.) on the Galapagos Islands, where hatching coincides with flushes of insect availability occurring after semi-seasonal rains (Hau et al. 2004; Hahn et al. 2008). However, in male Darwin finches, rainfall or even rainfall-related noise stimulates singing (Grant 1999) and may therefore act directly as a proximate cue for reproduction.

Direct evidence that vegetation phenology is likely to provide an environmental cue for breeding activity comes from red crossbills (*Loxia curvirostra*) that appear to breed in response to the changing availability of their main food, western hemlock (Hahn et al. 2008), and Piñon jays (*Gymnorhinus cyanocephalus*) that breed in late summer only if green cones of Pinon pines (*Pinus monophylla*) are abundant (Ligon 1974; Ligon 1978). The possibility that food acts as a phenological cue for breeding has been demonstrated in a study using captive male spotted antbirds (*Hylophylax n. naevioides*) in Panama, in which gonadal growth and singing were stimulated by the addition of live crickets to their diet, while singing was induced even when crickets were only presented visually (Wikelski et al. 2000). In addition, Perfito and colleagues showed in captive lesser sundas zebra finches (*Taeniopygia g. guttata*) that food availability, in the form of seeds, is a more potent stimulus than increasing day length in regulating testicular development (Perfito et al. 2008). It thus appears that opportunistic breeders use phenological cues alone or in combination with rainfall and/or temperature cues to time the onset of breeding.

Strictly seasonal breeders may also use phenological cues, such as bud burst and the associated appearance of invertebrate prey, to fine-tune the timing of breeding to local conditions, superimposed on an underlying seasonal reproductive pattern (Hahn 1998; Hahn et al. 2008), particularly if the seasonality of their environment has an unpredictable component (Wingfield et al. 1992). These cues may be dependent on changes in ambient temperature, for example in insectivorous seasonal breeders

Table 10.1: Review of selected publications reporting effects of spring phenology on the seasonal timing of reproductive development and egg laying in both (**A**) seasonal opportunists and (**B**) strictly seasonal breeders. gran.=granivorous, insect.=insectivorous, obs.=observational study, exp.=experimental study.

Bird species	Food	Cue	Behavioural or physiological measure	Study	Reference
A) Seasonal opportunists					
Piñon jays (*Gymnorhinus cyanocephalus*)	gran.	green cones of Piñon pines	breeding commences in summer when cones are present	obs.	Ligon 1978
Zebra finches (*Taeniopygia guttata*)	gran.	rainfall	hatching coincides with ripening of grass seeds after rain	obs.	Zann et al. 1995
Red crossbills (*Loxia curvirostra*)	gran.	cones of hemlock	breeding commences when cones are present	obs.	Hahn 1998
Darwin's ground finches (*Geospiza spec.*)	insect.	rainfall	breeding commences after rainfall	obs.	Hau et al. 2004
Darwin's ground finches (*Geospiza spec.*)	insect.	rainfall	rainfall-related noise stimulates singing	exp.	Grant 1999
Spotted antbirds (*Hylophylax n. naevioides*)	insect.	addition of live crickets to diet	faster testis growth when live crickets present	exp.	Wikelski et al. 2000
Spotted antbirds (*Hylophylax n. naevioides*)	insect.	visual cues of live crickets	increased song rates when crickets visible	exp.	Wikelski et al. 2000
Zebra finches (*Taeniopygia g. guttata*)	gran.	food availability, day length	faster gonadal growth when unrestricted food present	exp.	Perfito et al. 2008

Bird species	Food	Cue	Behavioural or physiological measure	Study	Reference
B) Strictly seasonal breeders					
Great tits (*Parus major*)	insect.	oak and caterpillar phenology	breeding coincides with oak and caterpillar phenology	obs.	Jones 1972
Great tits (*Parus major*)	insect.	oak and caterpillar phenology	breeding coincides with oak and caterpillar phenology	obs.	Perrins 1965
Great tits (*Parus major*)	insect.	tree phenology	breeding coincides with birch leafing	obs.	Slagsvold 1976
Pied flycatchers (*Ficedula hypoleuca*)	insect.	tree phenology	breeding correlates only weakly with vegetation phenology	obs.	Slagsvold 1976
Great tits (*Parus major*)	insect.	caterpillar phenology	breeding coincides with caterpillar phenology	obs.	Nager & van Noordwijk 1995
Great tits (*Parus major*)	insect.	tree and caterpillar phenology	breeding coincides with oak and caterpillar phenology	obs.	van Noordwijk et al. 1995
Great tits (*Parus major*)	insect.	oak phenology	no correlation between delayed oak bud burst and breeding	obs.	Visser et al. 2002
Song sparrows (*Melospiza melodia morphna*)	insect.	temperature, emergence of shoots	testis growth coincides with presence of vegetational cues	obs.	Perfito et al. 2004
Great tits (*Parus major*)	insect.	oak and birch phenology	breeding coincides with leafing phenology of oak	obs.	Nilsson & Källander 2006
Blue tits (*Cyanistes caeruleus*)	insect.	oak and birch phenology	breeding coincides with leafing phenology of birch	obs.	Nilsson & Källander 2006
Barn swallows (*Hirundo rustica*)	insect.	plant phenology	breeding coincides with leafing of elm and flowering of snowdrop	obs.	Møller 2008
Blue tits (*Cyanistes caeruleus*)	insect.	oak bud burst	breeding coincides with oak bud burst	obs.	Bourgault et al. 2010
White-crowned sparrows (*Zonotrichia leucophrys*)	gran.	sprouted wheat leaves	ovary, but not testis development advanced by food supplement	exp.	Ettinger and King 1981
Great tits (*Parus major*)	insect.	branches of pedunculate oaks	no correlation between development of branches and breeding	exp.	Visser et al. 2002
Great tits (*Parus major*)	insect.	branches of downy birch	luteinizing hormone rise accelerated in presence of branches	exp.	Visser et al. 2002
Blue tits (*Cyanistes caeruleus*)	insect.	branches of downy or evergreen oak	breeding advanced in presence of evergreen oak	exp.	Visser et al. 2002
Island canaries (*Serinus canaria*)	gran.	simulated rainfall or vegetation	rainfall induces rise in testosterone and advances breeding	exp.	Voigt et al. 2007

relying on a food peak in spring to rear their young (Kluyver 1952; Perrins 1965; van Balen 1973; Dhondt & Eyckerman 1979; Schmidt 1984; Perrins & McCleery 1989; Crick et al. 1997a; Visser et al. 1998; Sokolov 2000; Cresswell & McCleery 2003; Visser et al. 2003; Both et al. 2004). It is thus difficult to distinguish between direct effects of increasing temperature (Schaper et al. 2012) and phenological phenomena cues for timing the onset of breeding.

Possible phenological cues used by insectivorous seasonal breeders for reproductive timing have been most extensively studied in great and blue tits (*Cyanistes caeruleus*), which appear to respond to the leafing of trees and appearance of caterpillars (Table 10.1). Egg laying of great tits in Oxford, UK, is associated with oak bud burst and the first appearance of caterpillars (Perrins 1965; Jones 1972; van Noordwijk et al. 1995), while in some other European populations it coincides with the timing of birch (*Betula pubescens*) leafing (Slagsvold 1976). In Switzerland, laying dates of great tits correlate with the appearance of caterpillars in mixed forests of conifer, beech (*Fagus sylvatica*), oaks (*Quercus* spp.) and hornbeam (*Carpinus betulus*, Nager & van Noordwijk 1995). In Swedish coastal and inland habitats, variation in laying dates of great and blue tits correlates with leafing phenology of pedunculate oak (*Q. robur*) and birch (*B. pendula*, Nilsson & Källander 2006). In blue tit populations in Corsica, living in broad-leaved deciduous downy oak (*Q. pubescens*) or in evergreen Holm oak forests (*Q. ilex*), egg laying occurs at different times depending on forest type but, regardless of forest type, there is a close correlation between bud burst date and laying date (Blondel et al. 1993; Bourgault et al. 2010). In contrast to these studies, Visser and colleagues observed in great tits, in a 'natural experiment' in the Netherlands, that the bud burst of pedunculate oaks did not correlate with the onset of egg laying when bud burst was delayed in 1992 by an unseasonal frost in the previous year (Visser et al. 2002).

A close relationship between spring phenology and laying date has also been observed in some non-Parids. In song sparrows (*Melospiza melodia morphna*), in the Northwest USA, differences in the timing of reproductive development between coastal and montane populations can be best explained by an integration of temperature cues and vegetation cues in the form of fresh shoots (Perfito et al. 2004). In Danish barn swallows (*Hirundo rustica*), the onset of laying is also closely correlated with the phenology of local vegetation, such as broad-leaved elm (*Ulmus glabra*) and snowdrop (*Galanthus nivalis*), which is related to increased temperature (Møller 2008). In contrast with these studies, in the pied flycatcher (*Ficedula hypoleuca*), a migratory insectivorous passerine, seasonal vegetation phenology does not provide a strong cue for the timing of breeding (Slagsvold 1976, Table 10.1).

Only experiments under controlled conditions can answer questions about the causal effects of supplementary phenological cues on the timing of seasonal avian reproduction, and observations reported to date are conflicting. The timing of the onset of laying in captive great or blue tits kept in outdoor aviaries has been compared

in a multi-site experiment carried out in the Netherlands, Sweden and Corsica (Visser et al. 2002). In the Netherlands, the onset of breeding in pairs of great tits given leafing pedunculate oak branches was not affected by the stage of development of the leaves (Visser et al. 2002). In Sweden, gonadal growth and concentrations of plasma testosterone were the same in male great tits in the presence and absence of leafing branches of birch, although an increase in plasma luteinizing hormone (LH) was advanced in the presence of leafing branches (Visser et al. 2002). In contrast, in a study of captive blue tits from two populations in Corsica, provided with phenological cues from branches of downy oak , the most common tree in the habitat of one population, or of evergreen Holm oak, the most common tree in the habitat of the other population, the laying dates were advanced in both populations when they were provided with leafing evergreen oak (Visser et al. 2002). In a study on song sparrows from the Northwest USA, differences in the timing of the onset of laying in free-living birds observed at different altitudinal temperatures were not replicated in a laboratory study in which the birds were exposed to the same temperatures but not provided with phenological cues (Perfito et al. 2005). White-crowned sparrows (*Zonotrichia leucophrys gambelii*) receiving green leaves of wheat sprouts as a food additive for 20 days showed a significant increase in ovarian mass compared with controls, even though body mass and testicular mass were not affected (Ettinger & King 1981). Finally, in a study on wild island canaries (*Serinus canaria*) held under short day conditions, the onset of breeding was advanced after exposure to green grass (*Poa pratensis*), bamboo (*Phyllostachys aureosulcata*) and white spruce (*Picea glauca conica*), but not after exposure to simulated rainfall (Voigt et al. 2007).

Spring phenology could influence the onset of laying in several ways. Firstly, if the onset of reproduction is energy limited, the increase in prey abundance and diversity might provide energy and nutrient resources to build up reproductive tissues, thus facilitating an early onset of laying. The effect of food availability on the onset of breeding may be dependent on the appropriate ecological conditions (Bourgault et al. 2009). Pre-breeding food supplementation experiments in single-brooded passerines have produced ambiguous results, either showing no effect or advancing the onset of laying by no more than 1 week (Meijer & Drent 1999; Harrison et al. 2010, but see Scheuerlein & Gwinner 2002). Secondly, spring phenology could influence the onset of laying through changes in the composition of the bird's diet, adding chemical compounds that speed up reproductive development. This possibility is suggested by an observation in montane voles (*Microtus montanus*) in which testicular development and mating are stimulated by 6-methoxybenzoxazolinone (6-MBOA), a substance found in growing seedlings eaten by the animals in spring (Berger et al. 1981; Berger et al. 1987). It is possible that birds ingest similar secondary chemical compounds while feeding on buds of deciduous trees in early spring (Betts 1955), which might affect their reproductive system in a comparable way. However, Bourgault and colleagues investigated the oak bud scale remains in blue tit gizzards and concluded

that consumption of large amounts of buds does not occur before egg laying (Bourgault et al. 2006). Lastly, temperature-dependent vegetation and invertebrate phenology might accelerate the onset of laying by providing a visual stimulus that is translated into a reproductive neuroendocrine response, comparable to the effect of photostimulation in early spring (Hahn et al. 1997 ; Moore et al. 2006; Ball & Ketterson 2008; Stevenson et al. 2008). It thus seems likely that phenological cues providing a visual stimulus may be used to fine-tune the onset of reproduction in a photoperiodic seasonal breeder, such as the great tit.

In order to test the hypothesis that vegetation and invertebrate phenology might advance the onset of laying by providing a visual stimulus, it is first necessary to identify a suitable temperature-dependent cue and an appropriate measure of reproductive neuroendocrine response. If birds have evolved to adjust their reproductive timing in response to vegetational cues, these cues should reliably provide information on the future timing of an invertebrate food peak. After dormancy release, deciduous trees of mature forests, e.g. oaks, respond to increased photoperiod in spring, which is modulated by temperature. In contrast, many short-lived, early successional trees, e.g. birches, are primarily temperature sensitive (Körner & Basler 2010). The leafing of these trees marks the onset of spring in temperate zones (Chmielewski & Rotzer 2001) and is thus available to insectivorous birds as a cue integrating past temperature patterns and predicting the temperature-dependent hatching of lepidoptera caterpillars.

We experimentally investigated whether great tits make use of phenological cues from birch, as the leafing of birch branches coincides with the beginning of egg laying in great tits in the natural population used in our study (see Fig. 10.1 for details). The development of vegetation in early spring promotes an increase in invertebrate food sources, especially caterpillars feeding on developing leaves (Buse & Good 1996; van Dongen et al. 1997). Therefore, caterpillars were also presented as a visual phenological cue. It was predicted that reproductive development and onset of laying of breeding pairs provided with these supplementary cues would advance relative to control pairs. The causal reproductive neuroendocrine response was assessed in both sexes by measuring changes in the concentration of plasma LH, which correlates with increasing gonadal activity and the onset of breeding in blue tits exposed to natural lighting (Caro et al. 2006).

Materials and Methods

Experimental birds and housing

These experiments were carried out under licence CTE 09.08 of the Animal Experimentation Committee of the Royal Dutch Academy of Sciences (DEC-KNAW).

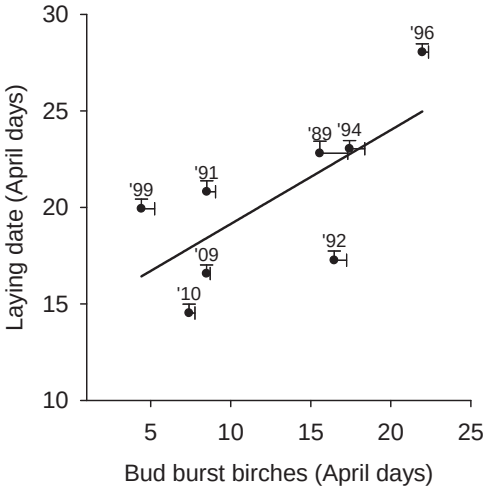

Figure 10.1: Laying dates of great tits of the Hoge Veluwe population in relation to birch (*Betula pendula*) bud burst dates, defined as the stage when green tips of leaves are unfolding. The analysis was restricted to years (see labels in the graph) in which at least 10 birches from long-term monitoring sites were scored twice a week (range 10-51 trees). The exact laying dates based on daily nest visits only encompass first clutches. If including the exceptionally late year 1992 (see the discussion of Visser et al. 2002 in the Introduction), the relationship is marginally non-significant (linear model, t1,7=2.34, P=0.058), while without 1992 bud burst predicts laying dates well (linear model, t1,6=3.32, P=0.021). Means ± standard errors are given. Note the advancement of laying in recent years (2009 and 2010) relative to the birch bud burst. Laying dates in April days, where 1 = 1st April.

Eighty great tits from a long-term study population at the Hoge Veluwe (The Netherlands) were taken into captivity as nestlings in 2008. Broods were selected from early- or late-laying maternal lines (Schaper et al. 2012). All chicks were blood sampled and sexed (Griffiths et al. 1998), and extra-pair offspring were identified (Saladin et al. 2003) prior to brood choice. On day 10 post-hatching, chicks were taken to the Netherlands Institute of Ecology (Heteren) for hand-raising (Drent et al. 2003). After independence they were kept in single-sex groups in open outdoor aviaries (2 x 4 x 2.5 m). The birds were fed *ad libitum* with a constant daily amount of food, consisting of a mixture of minced beef, proteins and vitamins, complemented by sunflower seeds, fat balls, a mix of dried insects (Carnizoo, Kiezebrink International, Putten, The Netherlands), proteins, vitamin and mineral supplements (Nekton S and Nekton Bio, NEKTON GmbH, Pforzheim, The Netherlands), calcium and water

for drinking and bathing. In December 2008, 36 breeding pairs were transferred to climate-controlled aviaries. During the 2009 breeding season the birds were kept under naturally increasing photoperiod and on an average temperature of either 14°C or 8°C, which did not affect the onset of laying (Schaper et al. 2012). The birds were moved back to outdoor aviaries in December 2009, kept in single-sex groups over winter and 16 pairs were reformed again in spring for their second breeding season in 2010. These pairs had bred together in 2009, except in two cases where the females were paired with a new mate as their original mates had died. Two pairs did not lay eggs in 2009, but bred successfully in the experiment reported here.

The breeding pairs were housed in two rows of outdoor aviaries from January 2010 onwards. One side of the aviary complex opened onto a grass field, while the birds from the other aviary row could see a hedge, mainly consisting of elder (*Sambucus nigra*) and hawthorn (*Crataegus* sp.) at about 15 m distance. In 2010, all birds were kept under natural temperature and daylight conditions. Lighting was supplemented by two tubular lights, which were on for 2 h after sunrise to compensate for the shading effect of the aviary roof. The aviaries offered a choice of four nest boxes. Moss as nesting material was provided from mid-February onwards.

Treatments

Pairs of birds were randomly and equally assigned to a control and a treatment group in the two rows of aviaries. To simulate an early onset of spring and the availability of prey in the environment, the birds were provided with leafing birch (*B. pendula*) branches and caterpillars. Phenological cues were added from 9th March until the end of May. During this period, day length increased from 11 h 18 min to 16 h 21 min. The cues consisted of branches that had been kept at room temperature for 1 week until an advanced bud burst occurred. Five branches about 1.5 m long with just unfolding leaves were provided for each breeding pair and replaced twice weekly. In addition, a covered transparent 20 cm Petri dish was placed on a feeding table in the centre of each aviary, containing about 20 caterpillars of the great cabbage white (*Pieris brassicae*) at larval instars 2-3 on a cabbage leaf. The larvae were replaced weekly, after they had developed into instars 3-4.

Birds from the control group received undeveloped birch branches with tightly closed buds and, for an equivalent cage enrichment, paper 'leaves' were added consisting of 4 x 4 cm red and blue cardboard squares placed over the branches. Control branches were rotated twice a week to simulate branch replacement and torn 'leaves' were replaced. As an equivalent to the presentation of caterpillars, small twigs, which could freely roll around, were placed in the Petri dishes. The birds made extensive use of both the birch branches with young leaves and the control branches with paper 'leaves' by climbing in them and pecking and destroying buds and leaves. It is likely

that birds from the treatment group regularly consumed buds and leaves. The birds were also attracted to the caterpillars and in a few cases succeeded in opening the Petri dishes to eat them. It was therefore concluded that the caterpillars provided a satisfactory food cue.

Measurements

Nest boxes were checked daily for eggs. The day that the first egg was found is referred to as the laying date. Blood samples of 100 µl were taken from the jugular vein every 2 weeks for LH analysis. Additionally, an initial sample was taken a week prior to the provisioning of phenological cues. Plasma was separated from red blood cells and stored at -80°C. Plasma LH concentrations were determined using a chicken LH radioimmunoassay (Sharp et al. 1987) validated for use in blue tits (Caro et al. 2006). The assay reaction volume was 60 µl, comprising 20 µl plasma sample or standard, 20 µl primary antibody (rabbit anti-chicken LH) and 20 µl of ^{125}I-labeled chicken LH. The primary antibody was precipitated to separate free and bound ^{125}I label using 20 µl of donkey anti-rabbit precipitating serum and 20 µl of non-immune rabbit serum. The samples were measured in a single assay, in duplicate. The intra-assay coefficient of variation was 6.4% for a high value plasma pool and 8.1% for a low value plasma pool and the minimum detectable dose was 0.15 ng/ml.

Statistics

Laying dates in 2010 were analysed with linear models in R 2.10.0 (R Development Core Team 2009), including phenology treatment, as well as laying dates of the female's and male's mother in the wild (a measure of genetic disposition for early or late laying) and laying date of the pair in the previous year as covariates. LH data were log transformed to achieve normality and analysed in general linear models for females and males separately. First, we tested whether initial LH concentrations differed between treatment groups. Second, we tested whether plasma LH concentrations increased over time. Third, we tested whether the seasonal change in LH following the addition of phenological cues differed between the groups in a mixed model with bird identity as a random factor (procedure lmer, package lme4). Fourth, we tested in a general linear model whether the rise in plasma LH 2 weeks after addition of phenological cues was different between treatment groups, as plasma LH concentrations can increase within days of exposure to a stimulatory cue (Meddle & Follett 1995; Wingfield et al. 1997). Explanatory variables were week of measurement (as a factor), phenology treatment and the interaction between the two. Fifth, we tested in a linear model whether LH concentrations at the end of April were related to laying dates.

Results

Initial LH concentrations did not differ between phenology treatment and control groups at the start of the experiment (females: $t_{1,16}$=0.15, P=0.88, males: $t_{1,16}$=1.23, P=0.24, Fig. 10.2). In both treatment and control groups, plasma LH increased with time (females: sampling week, χ^2_1=27.5, P<0.001; males: sampling week, χ^2_1=12.8 P<0.001). Two weeks after the start of the experiment, compared with initial values, the difference in LH concentrations was not affected by the addition of phenological cues (females: $t_{1,16}$=1.45, P=0.17; males: $t_{1,16}$=1.33, P=0.21; Fig. 10.2). However, while in females there was no interaction between the effects of treatment and sampling date on the increase in plasma LH (treatment*sampling week: χ^2_3=4.61, P=0.20; treatment: χ^2_1=1.48, P=0.22; sampling week: χ^2_3=22.3, P<0.001; Fig. 10.2A), in males there was a significant interaction (treatment x sampling week: χ^2_3=11.29, P=0.010). In males exposed to phenological cues, LH concentrations were already near their maximum in early spring, just after the addition of phenological cues, while concentrations in control males increased more slowly, with the steepest rise in late April (Fig. 10.2B). Females with higher LH concentrations at the end of April tended to lay earlier ($t_{1,14}$=–2.06, P=0.062, Fig. 10.3). One male of a pair given phenological cues died, and one female of a pair also given phenological cues died after laying her first egg. The remaining male was transferred to breed with the remaining female, which started

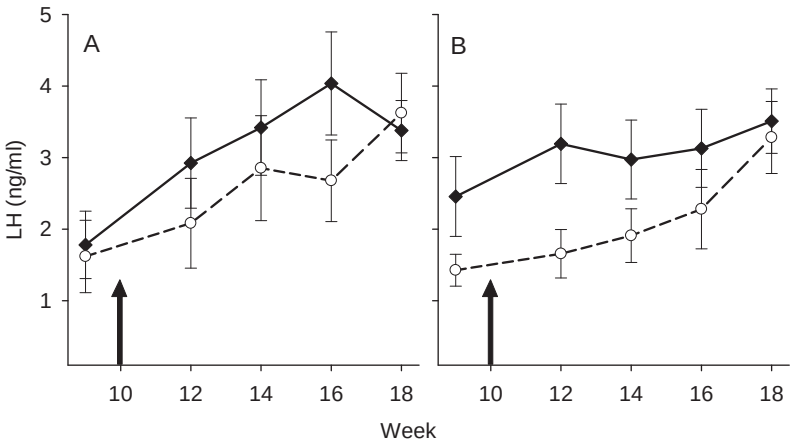

Figure 10.2: Luteinizing hormone (LH) development. LH concentrations measured in female (A) and male (B) great tits either with access to leafing birch branches and visual cues of caterpillars (filled diamonds, straight line) or with access to undeveloped branches and visual cues of pieces of twigs (open dots, broken line; control). Arrows indicate the addition of cues. Means ± standard errors are given.

laying 11 days later. Her laying date was included in the analysis. However, one female of the control group was ill and did not lay. Another female of the phenology group started laying extremely late on 11th June, which was considered to be too abnormal to be a consequence of the experimental design and was therefore excluded from subsequent analysis (Grubb's test for outliers: G=2.7, P=0.008). Laying commenced on 1st May, approximately 8 weeks after the birds were allocated to treatment or control groups. The onset of laying was not advanced by exposure to leafing birch branches and caterpillars (treatment: $t_{1,14}$=–0.40, P=0.71, Fig. 10.3). The genetic background of neither the female (laying date of female's mother: $t_{1,14}$=–1.38, P=0.20) nor the male (laying date of male's mother: $t_{1,14}$=1.68, P=0.13) influenced laying date. The onset of laying in 2010 in outdoor aviaries correlated with the onset of laying in 2009 in indoor climatized aviaries under standardized conditions ($t_{1,12}$=3.73, P=0.004, Fig. 10.4), which means that individual females laid consistently early or late in both years independent of supplementary cues.

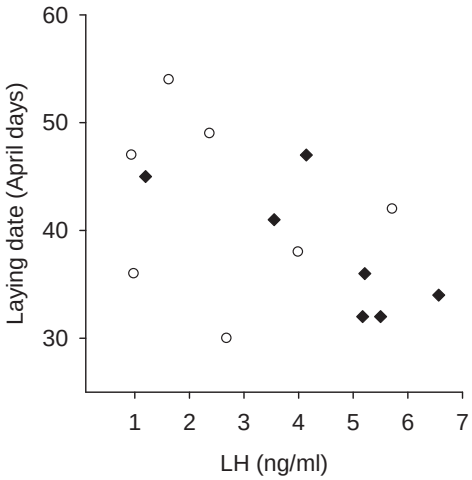

Figure 10.3: LH concentration and laying date. Relationship between female LH concentrations at the end of April (21st April) and laying date. Females with access to leafing birch branches and visual cues of caterpillars are represented by filled diamonds, while those with access to undeveloped branches and visual cues of pieces of twigs are represented by open circles. Laying dates in April days, where 1 = 1st April.

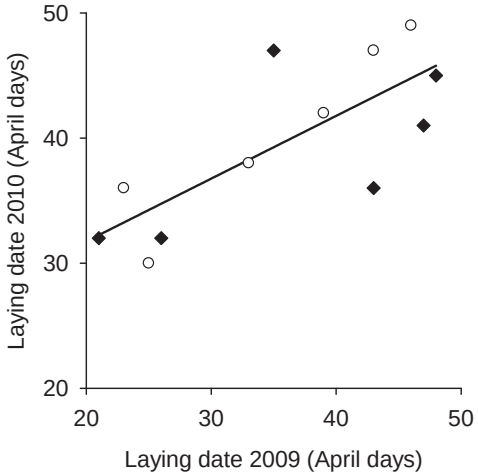

Figure 10.4: Relationship between laying dates in 2009 and 2010. Laying dates were recorded per pair of great tits breeding in climate-controlled aviaries in 2009 and in outdoor aviaries in 2010. Females with access to leafing birch branches and visual cues of caterpillars are represented by filled diamonds, while those with access to undeveloped branches and visual cues of pieces of twigs are represented by open dots. Laying dates in April days, 1 = 1st April.

Discussion

Vegetation phenology and food abundance have often been suggested as proximate supplementary cues in avian timing of reproduction, but there is little evidence for causality, especially in seasonal breeders. In the current experiment, great tits were exposed to phenological cues that are naturally present in their environment at the time of egg laying and are strongly affected by temperature. Contrary to prediction, exposure to leafing birch branches and caterpillars did not advance the onset of laying in great tits housed in outdoor aviaries exposed to natural light and temperature where birds had access to *ad libitum* food. This observation is consistent with an earlier study showing no effect of developing oak and birch branches on the timing of reproduction in captive great and blue tits (Visser et al. 2002). The lack of an effect of phenological cues in these earlier studies is therefore not a consequence of inhibitory cues associated with, for example, indoor caging. The failure to demonstrate an effect of phenological cues on the onset of laying is in contrast to many observations in free-living bird populations which imply, or suggest, that the correlation between either bud burst or food phenology and the onset of laying or reproductive activity is causal (see Introduction for references). The interpretation of earlier studies now requires critical re-assessment bearing in mind the following.

First, some experiments measure reproductive development without reporting laying dates in response to environmental cues. These experiments do not take into account the possibility that a given phenological cue may not affect ovarian development but instead the laying decision itself. This decision is made by the female (Caro et al. 2009), which may be responsive to supplementary cues that differ from those recognized by males (Ball & Ketterson 2008). In less favourable conditions than used in the present study, captive females often do not lay while males tend to show full gonadal maturation, which is why most experimental work has been restricted to males. The observation that females may not show full gonadal development under captive conditions indicates that cues additional to increasing photoperiod are required for the initiation of egg laying, which might be phenological or social cues. Researchers need to critically investigate whether the choice of physiological measures used to deduce changes in reproductive timing in response to a likely cue is appropriate.

Second, leafing date of, for example, the tree species hosting lepidoptera prey, or caterpillar emergence itself, is a standard phenological measure used to predict the timing of avian breeding (Table 10.1). Selection for synchrony with the food peak facilitates this correlation, but the bud burst of e.g. oak trees often commences late in spring, sometimes after the onset of egg laying, and therefore cannot be considered a predictive cue (Visser et al. 2002). It thus requires careful observation of natural systems to identify cues that are both relevant, in terms of predictability of future events, and timed in advance of changes in the phenological trait under investigation.

Third, the correlation between temperature, tree phenology and insect abundance excludes any inference of the causal relationship between any one of these cues and the timing of reproduction under natural conditions. Even though many studies report on relationships between phenological cues and laying dates (see Introduction), there is little experimental evidence for a causality, which should be a focus of future efforts.

In females, phenological cues did not affect the photoperiod-dependent seasonal increase in LH. In males receiving phenological cues, LH concentrations were coincidentally high from the beginning onwards, but did not increase much over time after the addition of cues. In contrast, control males showed a rise to levels similar to males from the treatment group over a period of two months. One can only speculate what would have happened if initial LH values in males from the phenology treatment group had been lower, but given the hormonal development in females we would not expect a difference between experimental groups.

Unfortunately, at the moment there is no available assay for avian follicle-stimulating hormone (FSH), the gonadotropin directly inducing follicle maturation, restricting researchers to measure LH instead. It is therefore possible that FSH and not LH is the mediator for the integration of phenological cues, but as here we found no effect of vegetation cues on the timing of laying itself, we would not expect different results for FSH.

From an ecological point of view, the functional significance of higher LH plasma concentrations in males exposed to predictive environmental cues in early spring is uncertain. As the development of the male reproductive system precedes that of the female, it is less likely that males will show an adaptive response to phenological cues to fine-tune gonadal development. Yet, in an opportunistic breeder, the rufous-winged sparrow (*Aimophila carpalis*), environmental factors associated with summer rains stimulated both gonadotropin-releasing hormone (GnRH) synthesis and LH secretion in males, which was, however, unrelated to gonadal growth earlier in the season (Small et al. 2008). Similarly, the higher LH concentrations reported by Visser and colleagues did not induce a greater increase in testis size (Visser et al. 2002); also, in the present experiment there was only a weak correlation between female LH concentrations and actual laying date. These findings demonstrate that different components of the hypothalamo-pituitary-gonadal axis might be influenced by various supplementary cues in different species. In addition, measuring the actual laying decision of the female is crucial to drawing conclusions about the timing of breeding.

As there was no effect of spring vegetational cues on the timing of reproduction in great tit females, it seems that the between-year variation in laying dates is triggered directly by temperature, which thus causes the correlation between birch bud burst and the onset of laying in the wild population (Fig. 10.1). In recent years, warmer springs have advanced both the leafing of birches and egg laying in great tits. The results of this experiment support our recent study (Schaper et al. 2012) showing that different patterns of increasing spring temperatures, rather than mean temperature itself, affect the onset of egg laying differently for early- and late-laying female great tits from the same population used in the current setup, implying genetic differences in sensitivity to temperature cues. The current experiment thus indicates that sensitivity to early spring vegetation, or food cues, plays only a minor role in fine-tuning the onset of egg laying.

Besides influencing the decision of when to lay, temperature can also affect the photo-induced timing of gonadal growth, as shown for white-crowned sparrows (Wingfield et al. 1997; Wingfield et al. 2003). To date, possible pathways that can accommodate this temperature effect, which might act at a physiological level or as a proximate cue, remain to be discovered. Low temperatures may also limit the speed of gonadal maturation by increasing the daily energy expenditure under natural conditions when food is scarce (Perrins 1970; Stevenson & Bryant 2000). In captive great tits, however, we did not observe an effect of ambient temperature on the regulation of gonadal growth (Schaper et al. 2012).

The high repeatability in the timing of laying between 2009 and 2010 in individual pairs, irrespective of whether they were early or late layers, supports findings by Visser and colleagues that laying dates of great tits in climate-controlled aviaries are

closely correlated with laying dates of the same females under natural conditions (Visser et al. 2009). This consistency again stresses a genetic component in the mechanisms underlying the timing of reproduction, which could well be sensitivity to environmental cues, such as photoperiod or temperature (Visser et al. 2011a), but is apparently not related to phenological cues.

In 2009, the birds in this study bred in climate-controlled aviaries (Schaper et al. 2012) and in 2010 bred again when exposed to more natural conditions in open aviaries. Against expectations, egg laying commenced later in 2010 than in 2009, even though second-year breeders normally lay earlier than first-year breeders and additional environmental information, also in the form of vegetational growth, was available to the birds in outdoor aviaries. Part of this effect could be attributed to the lower light levels caused by the roofing in the outdoor aviaries, as the increase in day length is the primary cue for the timing of reproduction. However, this is unlikely, as supplementary light was provided in outdoor aviaries. A different explanation could be that birds experienced colder night conditions in 2010 than in climate-controlled aviaries in 2009, which delayed the onset of laying relative to the previous year.

In conclusion, from both previous work and the experimental observations presented here, there is little direct evidence for an effect of tree phenology or presence of lepidopteran prey on the onset of reproduction in great tits. Nonetheless, several studies reported close correlations between tree phenology and laying dates of both opportunists and seasonal breeders in the field. Experimental work on a range of species is needed to further investigate whether those potential proximate cues assumed to advance or even induce breeding are really causal for the timing of reproductive development. This is one of a few studies that have examined direct effects of phenological cues on both male and female reproductive development, as well as egg laying under controlled conditions. More thorough physiological work concentrated on the reproductive development and behavioural decisions of the female is needed to investigate to what extent seasonal breeders make use of phenological cues. It is likely that, at least in great tits, the correlation between spring phenology and onset of laying is mediated by other proximate factors, such as direct temperature cues stimulating both vegetation growth and avian breeding.

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Chapter 11

Sonja V. Schaper, Peter P. de Vries and Marcel E. Visser

Variation in laying dates of great tits (*Parus major*) is linked to spatial variation in microclimate

Manuscript

Abstract

The reproductive season in great tits (*Parus major*) starts earlier in a warm spring. This behavioural plasticity in the onset of egg laying increases the synchrony of the breeding period with the availability of the main food sources needed to feed their young. However, there is clear within-population variation in the extend by which females shift their timing in warm springs. The mechanisms underlying this variation are not well understood and could come about because 1) females differ in condition, allowing only some females to start breeding earlier in warmer springs, as producing eggs early in the season is costly; 2) females differ in their sensitivity to temperature, causing more sensitive females to start breeding earlier in response to the same temperatures; or additionally 3) differences are caused by variation in microclimate between territories, causing females breeding in slightly warmer territories to lay earlier. To test the latter hypothesis, we employed a grid of temperature loggers in a nest box population of great tits. We interpolated ambient temperature at each nest box for a period in March/April identified as being relevant for the onset of laying across years. The spatial variation in the onset of egg laying was not negatively, but weakly positively correlated with spatial variation in ambient temperature. Between-female differences in laying dates within a year are thus partly caused by a delaying effect of higher temperatures on a spatial scale.

Introduction

Many small songbirds, including the great tit (*Parus major*), are known to start laying eggs earlier in a warm spring when the phenology of their environment is advanced (Kluyver 1952; Perrins 1965; van Balen 1973; Slagsvold 1976). By responding to the seasonal temperature increase (Schaper et al. 2012), female great tits can fine-tune their onset of egg laying and are thus able to exploit the earlier peak in invertebrate food abundance they rely on to feed their nestlings. Great tit breeding success strongly depends on the timing of breeding relative to the timing of the food peak (van Noordwijk et al. 1995; Verboven & Visser 1998; Visser et al. 2006). This food peak is mainly a result of the brief seasonal appearance of lepidoptera larvae (such as winter moths, *Operophtera brumata*). These caterpillars feed on the young leaves of oak trees (*Quercus* spp.) and their short peak in abundance is thus highly synchronized with the temperature-dependent bud-burst of the trees. In general, great tits advance egg laying in a warm spring, nevertheless, there is considerable within-year within-population variation in the onset of laying between females (Nager 1990). For example, in the great tit population investigated here the earliest and the latest laying date of a given year differ by about one month (see Results). While research has focused on the comparison of mean laying dates between populations (Slagsvold 1976; Lambrechts & Perret 2000; Visser et al. 2003; Both et al. 2004; Dunn 2004) or the investigation of long-term trends of particular populations (Crick et al. 1997a; Forchhammer et al. 1998; McCleery & Perrins 1998; Visser et al. 1998; Brown et al. 1999; Crick & Sparks

1999; Sokolov 2000), explanations for the variation in laying dates within a population are still largely unexplored (but see Nager 1990; Caro et al. 2009). This individual variation in the onset of laying could be explained by three factors: 1) differences in female condition, associated with differences in ontogeny, foraging ability or habitat quality, thus allowing females in better condition to start laying earlier in spring when conditions are still harsh (Visser et al. 2011b); 2) between-female differences in sensitivity to seasonal temperature cues, causing females to start breeding earlier or later in response to the same cues (Visser et al. 2011a; Schaper et al. 2012); or 3) microclimatic temperature differences between territories, causing females occupying slightly warmer territories to lay earlier. Here, we investigated the latter hypothesis in a population of great tits breeding in a heterogeneous woodland habitat.

Dhondt & Eyckerman (1979) observed that in a Belgian population of great and blue tits (*Cyanistes caeruleus*) a significantly larger amount of the earliest clutches in a year were laid by females breeding in warm nest boxes that were exposed to the afternoon sun, suggesting that indeed spatial variation in mean temperature could influence the onset of laying. Yet, previous experimental manipulations of nest box temperatures could not find evidence for an advanced or delayed laying date through heating or cooling (Nager & van Noordwijk 1992; Yom-Tov & Wright 1993). These studies concentrated on temperatures inside the nest box, which might bring energetic benefits to the female roosting in a warm nest box (Nord et al. 2011), therefore explaining the effect via the reduced energetic costs for early breeding. In contrast, in this paper we focus on small-scale variation in ambient temperatures in the nest box vicinity that could act as a supplementary environmental cue. As females spend only a short period per day roosting in a specific nest box, the ambient temperature in the immediate vicinity of the nest box, in which the female spends considerable amounts of time in the pre-laying period and which contains alternative roosts (Kluyver 1951; Dhondt & Eyckerman 1979) is likely a more adequate measure of the temperature the female is exposed to in the pre-laying period. Here we investigate if the natural spatial variation in ambient temperature between territories is associated with between-individual differences in the onset of egg laying.

Materials and Methods

Study area

The study was conducted in the Hoge Veluwe National Park (The Netherlands) in the years 2008-2010. The study area consists of about 400 nest boxes accessible for great tits that are distributed over 171 ha of mixed woodland. The vegetation is a mixture of pedunculate oak (*Quercus robur*), Northern red oak (*Q. rubra*), birch (*Betula* spp.), pine (*Pinus sylvestris*) and larch (*Larix* spp.) with some sparse undergrowth and interspersed with open heathlands. These vegetation features can create spatial

variation in ambient temperatures, both by shading during the day (from the time in springs that leaves are unfolding), storing heat during the night and by reducing wind speed. There are no relevant differences in elevation throughout the study area.

Temperature variation

To determine microclimatic differences in ambient temperature, sixty temperature loggers (iButton thermocrons DS1922 L, Maxim, Sunnyvale, USA) were placed in a regular grid over the area in close proximity to specific nest boxes (Fig. 11.1a-c). The iButtons were placed on the north-facing bark of a tree at a height of about 1.80 m above ground level and attached with a small custom-made plastic holder. The temperature loggers were distributed randomly over the sixty sites and their positions interchanged between years. They recorded ambient temperatures once every 30 minutes from mid-January onwards until June. The iButtons have a resolution of <0.06°C, but showed a consistent deviation to the true temperature. We therefore calculated the deviation for each logger by exposing them to three constant temperatures (5.5, 10 and 17.5°C) in climate-controlled cabinets and afterwards adjusting the measured temperatures in the field by the deviation of individual loggers from the mean of all loggers (±0.14°C at most). We calculated a daily mean temperature from the 48 temperature readings per day recorded by each logger.

To characterise the temperatures per nest box we used the fixed temperature period 13th March to 20th April over which we averaged ambient temperature as it is the between-year variation in temperature in this period which correlated best with the between-year variation in mean laying dates of the Hoge Veluwe great tit population for 1973 to 2010 (r^2=0.69, P<0.001, calculated by using a sliding window approach, see also Visser et al. 2006). Note that our approach of using the temperature mean of a fixed period means that for females starting to breed in the first half of April her laying date is correlated with a mean temperature that includes a period after she started laying. However, as we are merely interested in characterising the temperature deviation of nest boxes relative to each other, we do not perceive this as a problem.

Using inverse distance weighing (ArcView 9.3, ESRI), a spatial temperature map was interpolated for each year (Fig. 11.1a-c) from the 60 recording sites. A mean temperature over the measurement period was thus calculated per year for each of the 400 nest boxes.

Laying dates

To determine the onset of laying, nest boxes were inspected at least weekly, but often on a daily basis as part of the regular monitoring effort. If more than one egg was discovered in the nest box at inspection, the laying date was calculated by assuming that one egg was laid per day. Only laying dates of first clutches were included in the

analysis, thus excluding second clutches and replacement clutches (which includes clutches that were laid more than 30 days after the first clutch of a season was initiated). Both parents were caught when their chicks were seven days old. They were identified by individual metal rings and the female's breeding experience (first-time breeder or experienced breeder) was determined by using data from previous years. In case of unringed 'immigrants', their age was assessed by visual inspection of the wing coverts. When an unringed bird was not a first-time breeder it was allocated to a category of birds with unknown previous breeding experience. This category also included clutches that were abandoned before the female was identified. Thus three groups of clutches were formed: those from first-time breeders, from experienced breeders and from females with unknown breeding experience. If age or breeding experience play a role in the timing of the onset of reproduction (Forslund & Part 1995), we would expect experienced females to start laying earlier than inexperienced females.

Vegetation

In 2008, the composition of tree species and surface covered by tree foliage of certain species was determined for an area of 50 m radius surrounding each nest box as a measure of habitat quality. From these surveys, the total tree cover and the coverage of oak (*Q. robur* and *Q. rubra*) specifically around the focal nest box was calculated. As oaks are important host trees of caterpillars which are a major food source for great tit nestlings, they are therefore a determinant of habitat quality, mostly for the chick-feeding phase.

The vegetation measures were used in two ways: first, we analysed whether variation in total tree cover caused small-scale temperature variation. Second, we investigated whether in addition to the possible effects of vegetation on temperature the number of oak trees in the nest box vicinity had a direct effect on the timing of the onset of egg laying. We expected total tree cover to be negatively associated with ambient temperature. If the individual variation between females in the onset of laying is associated with differences in habitat quality (e.g. Wilkin et al. 2007; Sanz et al. 2010), more precisely with food availability in the nest box vicinity in late spring due to the presence of oak trees nearby the nest box, we would expect females breeding in nest boxes in a habitat that contains a large number of oaks to breed earlier.

All analyses were run in R 2.10.0 (R Development Core Team 2009). In a general linear model we determined the influence of small-scale temperature variation, the amount of oak trees present in the immediate nest box vicinity and the experience of the female on the timing of egg laying for each year. Additionally, the relationship between tree cover and temperature of the nest box vicinity was tested with a general linear model. Furthermore, we combined the data for all years in a mixed model (procedure lmer,

package lme4) that additionally included year (as a factor) and female identity fitted as random effect to account for multiple breeding events per female. In this model, temperature was standardized by substracting the mean and dividing by the standard deviation. We also tested the interaction between either standardized temperature and year or standardized temperature and mean temperature of a given year to determine if temperature effects differed between colder and warmer years. Additionally, the relationship between tree cover and temperature of the nest box vicinity was tested with a general linear model. We used a stepwise model reduction procedure to eliminate non-significant effects. In case of the mixed model, P-values were calculated by Markov Chain Monte Carlo sampling (function pvals.fnc package languageR). The results are presented including Bayesian 95% highest posterior density credible intervals, equivalent to 95% confidence intervals.

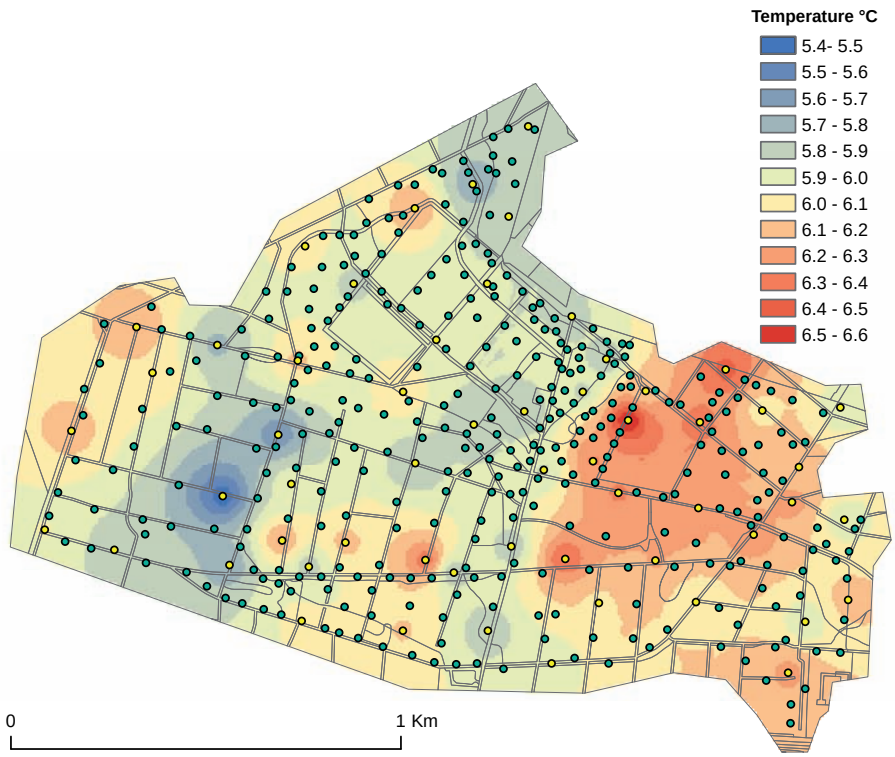
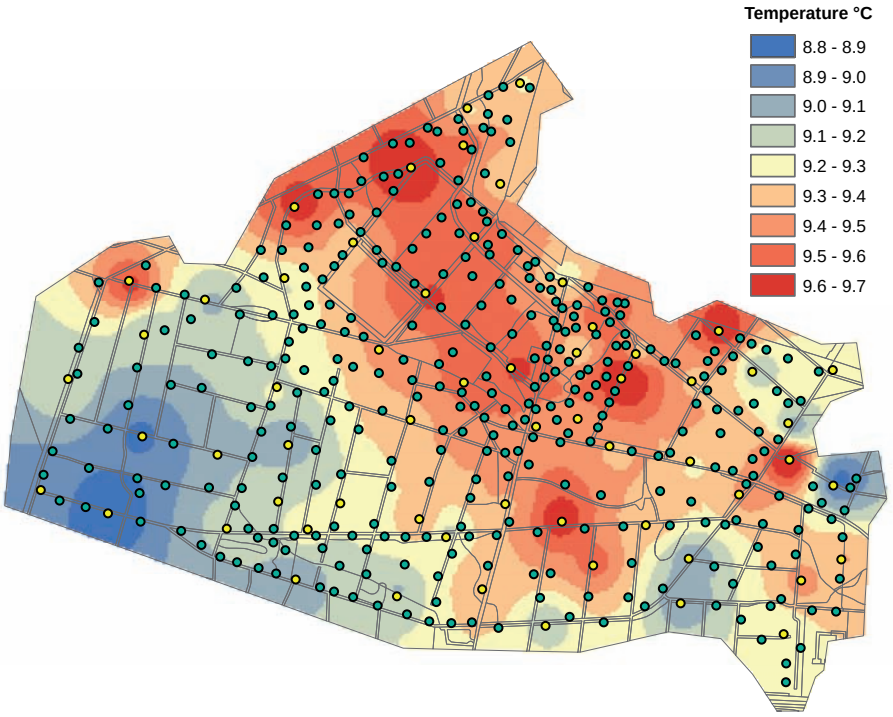
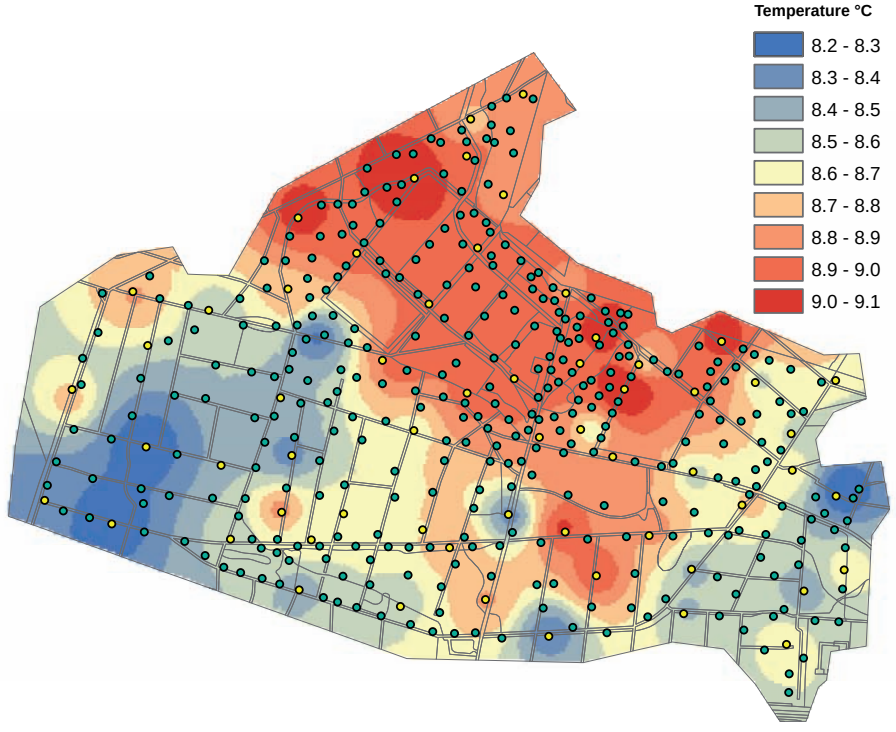


Figure 11.1a-c: Temperature variation over the Hoge Veluwe study area in 2008-2010. Interpolated map of the microclimatic variation in mean temperature for the period 13th March to 20th April. Direct temperature recordings were taken close to the 60 nest boxes marked in yellow. Note that temperature scales differ between years.


Temperature °C
8.8 - 8.9
8.9 - 9.0
9.0 - 9.1
9.1 - 9.2
9.2 - 9.3
9.3 - 9.4
9.4 - 9.5
9.5 - 9.6
9.6 - 9.7


Temperature °C
8.2 - 8.3
8.3 - 8.4
8.4 - 8.5
8.5 - 8.6
8.6 - 8.7
8.7 - 8.8
8.8 - 8.9
8.9 - 9.0
9.0 - 9.1

Results

The spring of 2008 was on average colder than in the two following years, as temperatures increased later over the period 13th March to 20th April (mean temperature 2008: 6.0°C, 2009: 9.3°C, 2010: 8.7°C). Over the 171 ha study area the microclimatic temperature variation in a given year fell within a range of about 1°C temperature difference (Fig. 11.1a-c). However, the temperature range of occupied territories was slightly narrower (2008: 5.7-6.3°C, 2009: 8.8-9.5°C, 2010: 8.2-9.0°C). In the two comparably warm springs of 2009 and 2010, specific cold and warm spots persisted, with the Northern part of the study area being slightly warmer and the South-western part showing particularly cold spots (Fig. 11.1b&c). In contrast, in the cold spring of 2008, especially the central Eastern part of the study area was relatively warm, while again the South-west was slightly colder than average. In contrast to the two warm years, also the Northern part of the study area was colder than average (Fig. 11.1a).

The total tree cover around occupied nest boxes, including both deciduous and coniferous trees, varied from 12 to 100% (mean 87%), while the cover attributable to oaks alone ranged from 0 to 96% (mean 32%). About two thirds of these oaks were pedunculate oaks (*Q. robur*), with a smaller amount of Northern red oaks (*Q. rubra*). Total tree cover did partly explain the variation in microclimate in the two warm springs 2009 and 2010 (both $P<0.001$, 2009: $t=-4.83$, 2010: $t=-4.68$), but not in the cold spring 2008 ($P=0.32$, $t=-1.00$) when bud burst of trees was delayed (personal observation).

In 2008, individual great tits started breeding between 13th April and 5th May (mean laying date 22nd April, 181 clutches), in 2009 between 8th April and 8th May (mean 17th April, 118 clutches) and in 2010 between 30th March and 30th April (mean 14th April, 121 clutches). Note that broods started more than 30 days after the earliest great tit laying date of the year were excluded from the analysis as they are considered to be replacement clutches. An equal number of breeding females were either young birds breeding for the first time (157 females) or experienced breeders of 2-6 years of age (167 females). For 96 birds previous breeding experience was not known, as they were either immigrants older than one year of age or not identified on the nest, mostly because they abandoned their clutch or brood in an early stage. 41 females bred in two years and 15 females in all three years.

The variation in the onset of egg laying was weakly, but significantly correlated with small-scale temperature differences between territories: females in warmer territories laid their first egg slightly later (Fig. 11.2, Table 11.1). This overall temperature effect did not differ between years (Table 11.1). However, when analysing each year separately, the positive correlation between ambient temperature and onset of laying was only found for the comparatively cold spring 2008 and not for the two warm

springs 2009 and 2010 (Table 11.2). The variation in laying dates was not associated with the amount of oak trees in the vicinity of the nest box or the breeding experience of the female, neither in an analysis of individual years nor for three years combined (Tables 11.1 & 11.2).

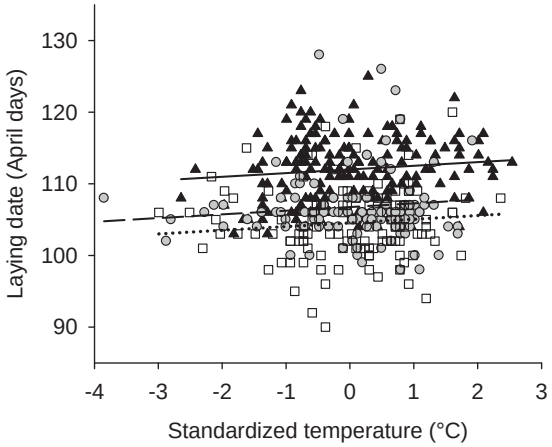


Figure 11.2: Relationship between the onset of reproduction in great tits and ambient temperature around the nest box. The laying date of the first egg in individual nest boxes is plotted against the mean temperature of the nest box vicinity over the period 13th March to 20th April in years 2008 (triangles and solid line) 2009 (circles and broken line) and 2010 (squares and dotted line).

Discussion

We found no evidence for a negative relationship between mean temperature in March/April and mean onset of egg laying in great tits, as may have been expected from such a correlation across years. In contrast, the influence of ambient temperature around the nest box on the laying date of individual birds was positive: females in relatively warmer territories laid their first egg slightly later. This indicates that either different mechanisms might act on a small spatial scale within a given year compared to a between-year time scale or that a third factor correlated with both temperature and laying date leads to this counterintuitive relationship.

In each of three years, the microclimatic temperature variation over the Hoge Veluwe study area was within a range of about 1°C between the coldest and the warmest nest box vicinity. Variation in the temperature pattern between cold and warm years could be caused by the vegetation structure: Deciduous trees will develop leaves and thus shade their surrounding earlier on in a warm year, thus influencing the microclimatic pattern differently in warm and cold springs in a mixed woodland. The spatial

Table 11.1: Results of an across-year analysis on the influence of ambient temperature (standardized per year), the percentage of oaks in the vegetation surrounding the nest box and the breeding experience of the female on the onset of laying in three consecutive years. Estimates for first-time breeders and experienced breeders are in comparison to females with unknown breeding experience. Female identity is fitted as a random effect.

Explanatory variable	Estimate	L 95% HPD	U 95% HPD	df	t	P
Temperature (standardized)	0.51	-0.03	0.90	1	2.20	**0.028**
% oak cover	0.003	-0.01	0.02	1	0.37	0.71
Breeding: experienced	-0.93	-2.49	-0.08	1	-1.52	0.13
Breeding: first-time breeder	0.58	-0.82	1.61	1	0.95	0.34
Year: 2009	-5.25	-6.41	-4.21	1	-9.80	**<0.001**
Year: 2010	-7.47	-8.43	-6.25	1	-13.80	**<0.001**
Temperature*year 2009	-0.67	-1.79	0.44	1	-1.20	0.23
Temperature*year 2010	-0.47	-1.48	0.65	1	-0.85	0.40
Temperature*mean temperature per year	-0.19	-0.51	0.13	1	-1.19	0.23

Table 11.2: Influence of ambient temperature, the percentage of oaks in the vegetation surrounding the nest box and the breeding experience of the female on the onset of laying in three consecutive years. Estimates for first-time breeders and experienced breeders are in comparison to females with unknown breeding experience.

Explanatory variable	Estimate	SE	df	t	P
			2008		
Temperature	5.49	2.45	1	2.24	**0.026**
% oak cover	0.012	0.013	1	0.94	0.35
Breeding: experienced	-0.49	0.81	1	-0.61	0.54
Breeding: first-time breeder	0.69	0.87	1	0.79	0.43
			2009		
Temperature	-0.49	3.41	1	-0.14	0.89
% oak cover	0.030	0.016	1	1.83	0.069
Breeding: experienced	-1.48	1.16	1	-1.27	0.21
Breeding: first-time breeder	0.98	1.10	1	0.89	0.37
			2010		
Temperature	3.66	3.24	1	1.13	0.26
% oak cover	-0.037	0.019	1	-1.90	0.060
Breeding: experienced	-2.31	1.47	1	-1.58	0.12
Breeding: first-time breeder	-0.30	1.45	1	-0.21	0.84

temperature variation present in the area is small compared to temperature changes from below 0°C to above 20°C that birds experience over springtime and to which they fine-tune their onset of laying (Visser et al. 2009; Schaper et al. 2012). The question is thus if this spatial temperature variation is large enough to have any relevant effect on the timing of egg laying. For a comparison, the maximum between-year difference in temperature over the same period is 4°C in our study area for the last 20 years, which leads to between-year differences in the population-mean onset of laying of about two weeks, or an advance of about 4 days per 1°C (S.V. Schaper, unpublished data). We could thus have expected a similar advancing effect on a small spatial scale within a year if mean temperatures were the relevant cues that birds respond to.

In the pre-laying period females forage in a larger area than the immediate nest box vicinity and will therefore be exposed not only to the microclimate around their nest box. Assuming a direct positive relationship between ambient temperatures in the nest box vicinity and the onset of laying could therefore overestimate the influence of these temperatures. Additionally, temperature was measured at 1.80 m above ground level, while birds forage mostly higher up in the canopy where the temperature range could be substantially different. Yet, we believe that females do spend considerable amounts of time close to their nest box (in their 'domicile', *sensu* Kluyver 1951) and are at minimum exposed to these temperatures during the night, as they roost in or close to the nest box. Also, temperature differences generally persist over larger parts of the study area (Fig. 11.1a-c), thus the temperature measured close to a focal nest box can be taken as being indicative of temperatures in an area that is accessible for the female to forage. However, the within-year difference between the earliest and the latest laying female is usually about a month, already illustrating that the small microclimatic temperature effect cannot explain this variation. In addition, our results oppose a negative influence of temperature on laying date on a spatial scale, as females breeding in a warmer territory laid later, rather than earlier.

In contrast to our findings, a significant negative relationship between small-scale temperature variation and the onset of laying was found in an earlier study on great tits breeding on a montane slope of 380-770 m altitude (Nager 1990): a temperature increase of 1°C corresponded to an advancement in laying of 5 days. With a temperature range of 4°C over the montane slope, the investigated variation was much larger than in the present study and the scale comparable to the temperature effect we find across years. However, the ten breeding sites situated along the temperature gradient also varied in altitude, exposure and forest type (mainly beech, *Fagus sylvatica*). Other factors correlated with this temperature gradient could thus play a role in influencing the onset of laying, therefore a direct comparison between the two studies is difficult.

In barn swallows (*Hirundo rustica*) the timing of leafing of broad-leaved elm (*Ulmus glabra*) and flowering of snowdrop (*Galanthus nivalis*) was correlated with the variation in the onset of egg laying between populations breeding at different sites (Møller

2008). However, in our study area small-scale variation in microclimate was neither associated with variation in bud burst of the dominant tree species (two species of oak, birch, larch and pine) measured at 36 sites (M.E. Visser & S.V. Schaper, unpublished data) nor variation in timing of the directly temperature-dependent biomass peak in caterpillars measured from droppings collected under these tree species (M.E. Visser & S.V. Schaper, unpublished data). Obviously, relying on small-scale temperature cues on a spatial scale would not be adaptive for female birds in our population, as these cues do not predict the timing of events in the future that determine great tit reproductive success, such as the timing of maximum food abundance.

That females started laying later in warmer territories is a puzzling result for which we lack an explanation. It is possible that the positive correlation between temperature and laying dates was partly caused by another factor not taken into account here. Even though we find a significant correlation, considerable variation in laying dates remains that is unexplained by microclimatic temperature differences (r^2=0.0005 for the regression line between temperature and laying date over all three years). More thorough research over multiple years with different temperature profiles needs to be conducted to identify a clear pattern in this relationship. However, ultimately temperature cues have to be uncoupled from other correlated climatic or phenological cues under controlled conditions to identify a causal relationship.

For great tits breeding in climate-controlled aviaries mean temperature was not perceived as a relevant cue for females determining their onset of laying (Schaper et al. 2012). Other factors are thus more likely to be responsible for between-female differences in the onset of laying in a given year. It has been suggested that this variation could come about through differences in female condition (te Marvelde et al. 2011; Visser et al. 2011b), causing females in worse condition to postpone their onset of laying to a period when energetic limitations are less severe, but it could also be caused by variation in the sensitivity to predictive environmental cues, such as temperature patterns (Visser et al. 2011a; Schaper et al. 2012). This would mean that females would breed earlier or later in response to the same seasonal temperature cues due to physiological differences in temperature perception and/or integration. Only studies that experimentally advance or delay females in their onset of egg laying under natural conditions and investigate fitness consequences of this shift can differentiate between these two causes. It seems that local microclimate plays only an insignificant role in explaining individual spatial variation in seasonal timing of reproduction.

Acknowledgements

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Chapter 12

General Discussion

Humankind currently experiences globally rising temperatures and changing weather patterns and one of the major challenges for ecologists nowadays is to assess how these changes will impact the natural environment surrounding us. By how much and in which ways will the communities of plants and animals that we value in our environment shift in composition? This is not only of interest for our own aesthetic sense, but can also have larger environmental and socio-economic implications. The most likely changes that we will experience in diverse ecosystems are range shifts and changes in seasonal timing, or phenology of species (Parmesan & Yohe 2003; Root et al. 2003; IPCC Core Writing Team 2007a). Much attention has been devoted to reproductive timing decisions of birds for a long time, which makes seasonal timing of reproduction a model phenomenon to study the impact of climate change on phenology. To date, proximate approaches to understand timing decisions have focused mostly on reproductive physiology, rather than egg laying itself, and the different biological disciplines studying avian seasonal timing are not yet sufficiently integrated (**chapter 2**).

In this thesis I investigated how environmental cues, such as ambient temperature, influence the phenology of the life-cycle of a small woodland songbird, the great tit. I studied how different stages and processes, ranging from photostimulated hormonal changes and gonadal development, to the onset and termination of egg laying, to the onset of moult, were affected by temperature cues. Using genetically related birds in experiments under controlled conditions allowed me to determine the extent to which the response to specific cues was genetically determined - a prerequisite for micro-evolutionary change. I observed the behaviour and physiology of birds in captivity and could thus not estimate fitness consequences of specific timing decisions under natural conditions. These, however, have already been demonstrated in wild great tits from the same population (Nussey et al. 2005; Husby et al. 2010). By examining timing decisions under controlled conditions I could establish a causal relationship between temperature cues and the onset of egg laying, which I could not have achieved under natural conditions due to the correlation between ambient temperature and the seasonal development of the abiotic and biotic environment. Investigating this relationship, as well as the interplay of environmental cues in the decision-making process of birds, is vital to determine how, and by how much, current climate change is disrupting the match between avian reproduction and their environment.

Temperature cues are critical for fine-tuning avian reproductive decisions

Birds of the temperate zone are well known to be phenotypically plastic in the onset of egg laying and reproduce earlier in warmer springs, synchronized with the peak abundance in their nestlings' food. Yet, few studies have gone beyond regressing mean or minimum temperatures of certain time periods against mean laying dates of a study population (for other approaches see Gienapp et al. 2005; Gienapp et al.

2010; van de Pol & Cockburn 2011). Our experiments using birds breeding in climate-controlled aviaries clearly demonstrate that mean temperature is not the relevant cue that great tits respond to, but rather the pattern of temperature increase, which is a signal to fine-tune the onset of egg-laying (**chapters 3, 4**). Additionally, the same relevant temperature cues led to a different behaviour in females from early- vs. late-laying families. Furthermore, in a wild great tit population we investigated the microclimatic differences in mean temperature in the immediate nest box surrounding pre-laying. Females that were exposed to the same seasonal temperature profile, but different microclimatic temperatures at any particular period of the season did only slightly adjust their laying date in response to this small-scale variation and in the opposite direction to what we predicted (**chapter 11**). Taken together, from these results I conclude that within-year differences in the onset of reproduction are rather a result of individual differences in temperature sensitivity than due to small-scale temperature differences. With an experimental approach using wild females, we could show that a short photostimulation in spring did not advance the onset of laying (**chapter 9**), again pointing to the importance of integrating secondary environmental cues in this decision-making process. Furthermore, phenology of the vegetation, such as the opening of leaves or the presence of caterpillars in the environment, was not perceived as a predictive cue (**chapter 10**). As the influences of these phenological changes are not distinguishable from influences caused by temperature patterns in a bird's natural environment, it was important to experimentally exclude the possibility that temperature patterns were perceived via spring phenology. I thus believe that phenotypic plasticity in timing of reproduction, which results in individual variation in the onset of laying in response to a shared environment in a given year, is determined by different sensitivities to predictive environmental cues, such as temperature patterns.

Evidence accumulates that not mean values of environmental variables themselves, but rather the patterns of change are important stimuli influencing behavioural decisions in birds. This seems to be true for temperature cues, but could also be the case for photoperiodic cues where, for example, a gradual seasonal increase in day length might have a more stimulating effect than a rapid change. This hypothesis was recently corroborated by experimental data: the gonadal growth of both female and male blue tits (*Cyanistes caeruleus*) was enhanced in response to a gradually increasing photoperiod, but not by a rapid change (S. Caro, unpublished results). White-fronted geese (*Anser albifrons*) have also been shown to respond more strongly to a change in an environmental cue rather than its absolute value. This species follows the flush of vegetation growth during migration to acquire the most nutrient-rich plants when feeding at spring stopover sites. In a recent study it has been observed that geese time their arrival according to temperature changes, more precisely, the changing acceleration of temperature (the 3rd derivative of the 'growing degree days'). This allowed the birds to arrive at a certain stopover site in advance of the maximum

increase in temperature, which in turn is directly related to the vegetation growth (van Wijk et al. 2011). Our findings how female great tits respond to temperature increases in timing the onset of reproduction is a similar example and adds to the growing evidence that birds integrate complex temperature cues directly to make predictions on subsequent changes in spring phenology. The next step to support these findings will be to validate them under natural conditions. A close investigation of temperature patterns pre-laying for different populations of wild great tits in different years is possible by using existing long-term data of breeding parameters and temperature patterns.

A critic might argue that these conclusions are drawn from experiments under highly artificial conditions. Yet the standardized rearing and housing conditions, with breeding pairs under *ad libitum* food conditions and not exposed to social interactions with conspecifics or environmental cues other than the lengthening photoperiod and artificial temperature treatments were pivotal to prove a causal relationship between temperature and reproductive timing. Nonetheless, they might have resulted in unnatural breeding behaviour. For example, the forced pairing of birds did not allow for free mate-choice by the female, which I believe to be the main reason that some pairs did not initiate breeding. The decision to start breeding or not was unrelated to the specific temperature treatment the pair was exposed to in any year and as I believe that the pair composition was the reason that some females did not start laying, these data points were not included as censored data in our analysis. Besides these difficulties of some birds not starting to breed, laying dates obtained from females breeding in climate-controlled aviaries were comparable to laying dates in previous (or following) years under more natural conditions (**chapter 10**, Visser et al. 2009), and I am therefore convinced that the variation in laying dates is not an artefact of captive conditions.

As with most scientific endeavours, our discovery that female great tits respond to the change in cue value rather than the value itself opens up many new intriguing questions, in addition to answering others:

1) What is the physiological mechanism that allows for the integration of complex temperature cues? 2) How do moderate temperature differences affect components of the hypothalamo-pituitary-gonadal axis that precede egg-laying? 3) Does the responsiveness to temperature cues change over time, or with reproductive state of the female? 4) Is cue sensitivity heritable? 5) Are there adaptive population-level differences in how temperature cues are integrated? 6) How is diversity in cue responsiveness maintained? 7) Most importantly, is there potential for natural selection to alter cue sensitivity in the face of climate change? 8) How is a shift in the onset of reproduction affecting other components of the life-cycle, such as the onset of moult, which might also become mismatched to local conditions? Answering these questions requires investigating temperature patterns, reproductive physiology and onset of laying on a population-wide, but also individual-based scale.

Reproductive physiology is mainly driven by photoperiod

In contrast to the onset of egg-laying, we showed that the underlying reproductive physiology is not regulated by changes in spring temperature patterns or moderate differences in mean temperature (**chapters 3, 4**). It is well known that photoperiod is the initial predictive cue driving the onset of reproductive development, but with respect to supplementary cues so far mostly constant extreme temperatures have been investigated in controlled experiments (Wingfield et al. 1996; Wingfield et al. 1997; Wingfield et al. 2003; Dawson 2005a; Perfito et al. 2005; Caro & Visser 2009). Even though the temperature patterns employed in our experiments are not directly reflecting a natural situation, they allowed the comparison between physiological responses to different more subtle temperature changes. By using moderate temperature differences we did not find a clear-cut effect of temperature cues on either endocrine or gonadal development. Moreover, as substantial differences between female follicle sizes were not related to the timing of egg laying, late stages of exponential follicular growth are apparently adjusted in response to non-temperature supplementary cues, or their maturation can be halted at a late developmental stage to fine-tune the onset of laying (**chapter 5, 9**). We did not investigate this possibility, as it would have been necessary to measure gonadal size repeatedly close to the (beforehand unknown) laying date, thus possibly also interfering with the laying decision.

A serious limitation in investigating reproductive physiology in small passerines is the comparably large amount of blood needed for hormone assays that does not allow for fine-scaled sampling. Due to this restriction, much temporal detail is lost in a monthly sampling scheme, and therefore our conclusions about endocrine changes in response to experimental temperature treatments (**chapters 3, 4**), but also spring phenology (**chapter 10**), need to be interpreted in light of this coarse temporal resolution. Hopefully future refinements in methodologies will allow a continuous sampling design, leading to a more detailed pattern. With more specific hormone assays available, more focus should also be devoted to the daily pattern in hormonal changes in relation to seasonal patterns.

The dissociation between reproductive development and the timing of laying itself remains a paradox to date. The missing connectivity gives rise to two basic questions: first, how does individual variation in the timing of gonadal growth come about and does it have adaptive relevance? Second, will advancement of the onset of laying at some point be restricted by incomplete reproductive maturation (see also **chapter 6**)?

The late stages of gonadal maturation are particularly interesting as the extent of early maturation apparently is not directly related to the onset of laying. It would be very worthwhile to investigate if fully-matured ovarian follicles can be maintained for a

number of days by the female, or if the rate of maturation itself can be adjusted in later developmental stages to fine-tune the laying of the first egg to the local environment. Again, more sophisticated methodologies are needed to answer these questions, such as possibly the assessment of follicle size without laparotomy. As we show that gonadal development does not predict laying dates well, future experiments will hopefully take actual laying decisions into account when determining the influence of environmental cues on reproductive timing.

Food availability determines investments in eggs

The timing of reproduction in birds is inevitably related to other life-history decisions, such as the number of clutches produced over the season, as well as clutch size or egg size per individual egg, which varies over the season. These decisions have been carefully investigated under natural conditions also for the great tit (van Noordwijk et al. 1981; Nager 1990; Nager & van Noordwijk 1992; Nager & Zandt 1994; Perrins 1996; Nager et al. 1997; Encabo et al. 2002; Pendlebury & Bryant 2005), but a direct influence of temperature on egg investments under controlled conditions independent of food abundance has, to my knowledge, never been determined in non-domesticated birds. We demonstrated that egg size in great tits did not increase, but rather decreased, with higher mean temperatures pre-laying (**chapter 7**). This means that the positive relationship that has been repeatedly established between temperature and egg size under natural conditions is in fact an indirect influence. Temperature drives prey abundance and detectability (Avery & Krebs 1984; Valtonen et al. 2011) and also determines energy expenditure in great tits (Stevenson & Bryant 2000; te Marvelde et al. 2011). Temperature is thus highly correlated with energetic and nutrient resources for egg production under natural conditions. In contrast, our results demonstrate that temperature is not taken into account as a predictive cue. Furthermore, we established that (inter-individual) egg size variation was substantially larger under controlled conditions, which clearly shows that energy-limitation is not the only cause of egg-size variation. As sisters were more similar to each other in the amount of egg investments than non-related females even under controlled conditions, these genetic differences in egg size are unrelated to heritable differences in foraging abilities or overall body condition. However, a high degree of flexibility in (heritable) variation in egg size still remains to be explained. Two related avian life-history traits, timing of egg laying and investment in individual eggs, are thus driven by different environmental components, temperature patterns and food availability.

Heritable variation in cue sensitivity and early reproductive physiology facilitates micro-evolution

A shift in timing of reproduction in response to directional climate change can be achieved by three interacting mechanisms: first, by the existing phenotypic plasticity in response to temperature, second, by a genetic shift in temperature sensitivity underlying this plasticity, and third, by a shift in the mean trait value in response to temperature. The existing phenotypic plasticity, however, is probably only functional within certain limits (Visser 2008), and as a species' 'climatic envelope' (Thomas et al. 2004) is shifting, the possibilities of genetic change in mean trait values gain more importance. In this process, phenotypic plasticity will likely only allow for short-term persistence under changing conditions and thus provide a time-buffer for micro-evolutionary changes to take place. When we thus want to evaluate the potential for micro-evolutionary change in the onset of reproduction of great tits, heritable variation in the physiology underlying the determination of laying date in response to environmental cues is crucial. This variation can include the physiology of the sensory system, but also the effector systems, including gonadal growth and egg maturation. In our experimental setup we specifically chose to use related individuals to assess whether the physiology underlying reproductive timing had a heritable basis. We found that family-members resembled each other in both the onset and termination of reproduction (**chapters 3, 4**), but also in the amount of egg investments (**chapter 7**). Heritabilities for (plasticity in) laying date (Sheldon et al. 2003; Nussey et al. 2005; Gienapp et al. 2006; Charmantier et al. 2008; Caro et al. 2009; Husby et al. 2010), but also egg size (van Noordwijk et al. 1981; Christians 2002) are known for great tits under natural conditions. To date, however, I am not aware of any study investigating the heritability of reproductive physiology in wild birds. We showed 73% of the phenotypic variation in the photoinduced extent of gonadal growth had a genetic basis in female great tits during early reproductive development (**chapter 6**). This heritable variation in different reproductive traits indicates that in a scenario where climate change requires an advancement of egg laying by more than the two weeks observable nowadays, micro-evolution in the underlying physiological mechanisms is possible.

Ideally, the extent of gonadal growth in winter should be determined repeatedly in (related) free-living birds under natural conditions to make predictions about the potential for micro-evolutionary change. However, this would involve an enormous effort, especially catching birds repeatedly in the non-breeding stage, and especially under winter conditions the laparotomies could pose a threat to the birds. A better solution could be to keep related, hand-raised birds in outdoor aviaries to assess their gonadal development in response to natural climatic conditions. However, gonads are thought to be regressed in winter to reduce maintenance costs, both from carrying reproductive organs around and maintaining them, but also from the increased predation rates a less versatile individual with larger gonads might face. In a captive

situation, where an individual does not pay these costs, gonadal growth could thus be released from an important energetic constraint. However, as we see that our great tits in climate-controlled aviaries do not grow their gonads earlier than wild birds, and probably even a bit later, gonadal maturation is probably not critically energy-restricted under the current conditions. I am thus convinced that we would have found a similar extent of gonadal growth, and same similarities between sisters, if these birds were wild-caught. Concerning the heritability estimates, the fact that the birds were hand-raised under standardized conditions reduces common-environmental effects and thus similarities between related individuals. In contrast, maternal effects in the form of egg investments, incubation and feeding in the first days of life are not accounted for. Therefore, the observed measures of variation and heritability in reproductive physiology and cue sensitivity are relevant, but not perfect, estimates for the possibility of a micro-evolutionary response.

Knowledge on the perception and integration of temperature cues relevant for seasonal timing is still rudimentary

Most physiological or (neuro)endocrinological approaches to seasonal timing are focused on the perception of photic cues and consequently the basis underlying seasonal photosensitivity is well known (Dawson et al. 2001; Hut 2011). In contrast, the pathways underlying an integration of non-photic environmental cues is still debated, even more since temperature cues which affect the onset of laying do not seem to affect the underlying reproductive physiology in terms of the seasonal rise in reproductive hormone concentrations or gonadal maturation (**chapters 3, 4**). However, adaptation of phenotypic traits, such as the onset of egg laying, can only be achieved if the mechanisms of physiological regulation that shape them, including the detection of cues, are changing in response to natural selection. Thermoreceptors responsive to ambient cold and warm temperatures have been identified in mammals and due to their highly conserved makeup can be expected to function in similar ways in birds. Yet, most research on the integration of temperature stimuli and efferent pathways is solely focused on thermoregulatory mechanisms. Therefore we still know little about the perception of gradual temperature changes within a range of 'comfortable' ambient temperatures. While the integration of temperature information at the level of the hypothalamus is well known with respect to thermoregulation, the ways in which these moderate temperature cues can affect the timing of seasonal events is speculative. Thyroid hormones have been suggested to play a role in mediating temperature cues in the context of seasonal reproduction, either by regulating energy metabolism, by eliciting morphological changes in gonadotropin-releasing hormone (GnRH) neurons or by direct sympathetic activation at the level of the ovary (**chapter 8**). At the current stage, physiologists need to realize that temperature cues are essential drivers of seasonal timing also for homeothermic organisms. The identification of direct effects of ambient temperature on seasonal

timing of reproduction might initiate more interest in investigating the mechanisms behind temperature perception and integration in birds.

Climate change will affect temperature patterns and thus the synchrony between great tit reproduction and the phenology of their prey

The ultimate goal of this research project was to obtain insight in the extent and the routes by which climate change will affect reproductive timing in great tits in the near future and how birds may adapt to a changed climatic situation. Possible scenarios for a given population include shifting their reproductive timing to restore the synchrony with the peak of food abundance, either via micro-evolution within the current gene pool or via replacement with genotypes from other climatic regions due to range shifts. However, as for instance photoperiodic conditions will not change in a given location, the new combination of abiotic conditions might not have existed previously somewhere else (Visser et al. 2009), so that it seems unlikely that a population is pre-adapted to the new local conditions. The two main abiotic factors influencing reproductive timing, photoperiod and temperature, will be phase-shifted to each other, as temperature rises will occur earlier in the year and thus under a different rate of photoperiodic increase. This means that birds, no matter where they originate from, have to adapt to a scenario that they never encountered before and thus have to change the mechanisms underlying their timing, or in other words, they have to change their responsiveness to environmental cues, such as temperature, or make use of different cues that predict future conditions better.

Climate scenarios roughly predict that in central Europe especially February and March temperatures will increase. For the last 30 years, however, mean temperatures during mid-April/May increased more than mean temperatures during March to Mid-April (values taken from the Royal Netherlands Meteorological Institute, KNMI). Yet, it is questionable if a temperature increase of 1°C in March is similar in its effect on a bird compared to a 1°C increase in April and how the temperature patterns underlying these temperature averages change precisely. As most climate models produce estimates of mean temperature change and only indicate an increase in 'extreme temperature events' (IPCC Core Writing Team 2007a), it is difficult to make predictions about how these changing temperature patterns will influence the connectivity of food chains and communities that are relevant to us.

Do experiments on temperature effects on reproductive timing thus help us understand how the great tit breeding season will look like in 2050? Clearly, they contribute to our understanding of how birds react to certain environmental cues; indeed we have shown for the first time that temperature patterns affect laying dates directly and not through the phenology of vegetation or food abundance. We have also shown that there is genetic variation in cue sensitivity (**chapters 3, 4**)

and in the timing of reproductive development (**chapter 6**), which will influence the patterns of micro-evolutionary change. These are important first steps to predict the impact of global warming on small woodland songbirds. Certainly, there are also other possibilities for these birds to adapt to changing conditions, such as the shift to alternative food sources to feed nestlings, and thus the dynamics of food sources are another unpredictable component in the estimation of population responses to climate change. In addition, the approach focusing on the contribution of predictive environmental information to seasonal timing does not take energetic costs of free-living birds into account that might prevent breeding at an earlier date.

A complementary approach: laying too early poses energetic costs that outweigh the benefits of matching the food peak

Complementary to my own work, Luc te Marvelde studied energetic costs of reproduction in free-living great tits from the Hoge Veluwe population. In this section I shortly summarize the main results from his thesis, entitled 'Coping with climate change: Energetic costs of avian timing of reproduction' (te Marvelde 2012).

After measuring availability of insectivorous prey and monitoring foraging behaviour in the pre-laying period, te Marvelde investigated whether timing of reproduction is constrained by food abundance, more precisely protein availability, thus allowing only females in good condition to breed early. He compared the daily energy expenditure (DEE) of early-laying females with the DEE for late-laying females and found no evidence for differences between early- and late-laying females. In addition, te Marvelde also investigated DEE at two stages during the chick-feeding period, when the female's chicks were 6 and 14 days old, but under a standardized foster brood of eight 10-day-old chicks. He found that even though females differed consistently in energy expenditure during chick feeding, the variation was not related to energy expenditure of the same females during egg laying. During chick feeding, DEE increased with lower temperatures, lower food availability and higher brood visit rates. DEE increased between the first and second measurement for late-laying females, but decreased for early-laying females, as early-laying females bred when food was still abundant on both occasions, whereas food conditions quickly deteriorated for late-laying females. Foster broods gained less weight during the second than during the first measurement period, even though females worked harder to feed the brood and even compromised their own condition during the second measurement period.

Finally, te Marvelde explored the hypothesis that being mismatched to a seasonal food peak could be an adaptive strategy. This could be optimal in a situation where temperatures in late spring increase disproportionally compared to early spring, causing the food peak to advance without lifting energetic costs of early egg laying, a hypothesis that was explored using a mathematical model. In a literature review,

a mismatch between avian timing of reproduction and the phenology of the food source was found in about half of the cases. This mismatch had mostly negative fitness consequences. In an attempt to test the 'adaptive mismatch hypothesis', for which females have to be manipulated such that they lay earlier than the controls, te Marvelde found that neither additional photostimulation nor a treatment with leptin, a hormone correlated with body fat content which signals body condition in mammals, could advance the laying decision of wild birds.

In summary, te Marvelde's work emphasized the female great tit's flexibility in foraging strategies, which allows a plastic onset of reproduction. He demonstrated that early- and late-laying females do not differ in their energy expenditure during the egg-laying period. This is not necessarily in contrast with the hypothesis that early laying is energetically constrained, as females might have chosen to breed early or late in response to their energetic state. That daily energy expenditure increased only for late-breeding females over the chick-rearing phase shows that early breeding per se is not important in terms of energetic costs, but that the synchrony with the food peak is essential. In my opinion, this synchrony is achieved by the fine-tuning of the onset of egg laying with the help of supplementary environmental cues.

Environmental cues affect avian reproductive timing on multiple levels

Temperature has a strong direct effect on the phenology of temperature-sensitive trees (Körner & Basler 2010), as it influences the timing of bud burst and thus the phase when young palatable leaves are available to insect herbivores. Direct temperature effects on the hatching date of winter moth larvae, which are an important prey species for great tits feeding nestlings, are even more pronounced (van Asch et al. 2007), such that the peak in caterpillar biomass is strongly advanced under climate change (Visser & Holleman 2001). Temperature thus determines the environment of selection for great tit parents indirectly through the phenology of their food. What is important in this respect is that stable moderate spring temperatures most likely have the same effect as fluctuating temperatures on the caterpillar phenology, but not on great tits. In the case of invertebrates, because we know the direct relationship between temperature and growth rate it is possible to predict a future phenological situation, based on the best prediction of rising mean temperature given by the IPCC. The egg laying decision of a female great tit is, however, a process that is fine-tuned by multiple environmental cues. Gonadal growth seems to be predominantly regulated by the highly predictable changes in photoperiod and photostimulation sets a broad timeframe for the onset of reproduction. The optimal period to reproduce is further refined by supplementary cues derived from temperature patterns, which, at least in the past, reliably predicted the optimal timing of laying relative to the caterpillar food peak. Direct effects of energetic costs possibly come only into play in the final days before laying, as some individuals that are unable to gather sufficient

(protein) resources might delay egg laying slightly. The interplay of these regulatory components thus makes a prediction of the onset of reproduction tricky. Nevertheless, we know that the major environmental component that drives the phenology of the oak tree-winter moth-great tit food chain is ambient temperature. Inconveniently for our estimation, different aspects of 'temperature' affect various components and at different times of spring. This makes a final estimation of the impact of climate changes in this shifting system highly speculative. As effects of moderate temperature changes in spring on other parts of the avian life-cycle, such as the onset of moult, are less pronounced (**chapter 3**), these might not shift in a similar speed or even in a different direction. Whether the relative shift of different life-cycle stages to each other in a changing environment has positive or negative consequences on the fitness of an individual bird is uncertain.

Synthesis

In this project I integrated a proximate, physiological approach to avian seasonal reproduction with a framework of evolutionary ecology. While the focus in avian timing has traditionally been on tracking changes in mean laying dates over time, I investigated how individual decisions are affected by environmental cues, such as temperature. Based on experiments under controlled conditions, I also estimated the potential for a micro-evolutionary change in the underlying reproductive physiology in response to long-term directional change, which is crucial to estimate population-level resilience to climate change.

I set out to explain the lack of an advancement in reproductive timing of great tits under contemporary climate change, which leads to a mismatch with the peak in food biomass. In this joint project on seasonal timing, I focused on individual differences in the reaction to supplementary cues, while Luc te Marvelde studied energetic costs. As pointed out earlier, these two approaches do not necessarily need to be contradictive, even though they take a different perspective. We have observed that individual female great tits are remarkably consistent in their laying pattern relative to the population mean under different environmental conditions, reflecting different degrees of phenotypic plasticity, and similarities between related females indicate that traits underlying the laying date of the first egg have a heritable component. We could show that great tit females differ genetically in their response to temperature cues, namely the pattern of temperature increase. As early- and late-laying females in our study population do not differ in the energetic costs of egg laying, the use of these supplementary cues to fine-tune laying behaviour is clearly connected to energetic demands during the late chick-rearing phase, where synchrony with the food peak is crucial to reduce the parental workload and possibly prevent malnutrition of the chicks. However, in recent years most females breed too late relative to the caterpillar food peak, and thus with the knowledge we gained from experiments

under controlled conditions, but also trying to manipulate the onset of breeding under more natural conditions, we are now better aware that we need to monitor changes in the seasonal temperature profile responsible for the apparent mismatch. Predictive cues are highly context-specific, but the use of cues to predict a future environment of selection is a general principle that is found in many taxa. Insights gained from the close observation of changes in the use of environmental cues over time in a small songbird can therefore be applied in other contexts. We should see the current situation in which we possess a wealth of information about the regulation of avian phenology as a unique opportunity to closely observe and understand evolution in action.

Appendix to chapter 3

Appendix 3.1 Proportional hazards model

The onset of reproduction as well as the termination of reproduction can be viewed as a "time to event" and analyzed with survival analyses, in this case using mixed effects Cox proportional hazards models (Cox 1972). Proportional hazards models can be used to analyse the probability that an animal will start laying at a particular day as a function of an unspecified base line hazard (Kalbfleisch & Prentice 2002) multiplied by a number of explanatory variables, which include fixed as well as time-dependent variables (see below). This approach has been applied for timing of reproduction and migration of wild populations (Gienapp et al. 2005; Bauer et al. 2008; Gienapp et al. 2010).

The advantage over the 'classical' approach of regressing population means or individual observations against an annual mean temperature measure is that one does not need to arbitrarily specify a fixed time-period of interest, such as monthly averaging of temperatures for example. Instead, the proportional hazard model allowed us to use an iterative procedure, based on a linear predictor, for the calculation of a temperature variable that incorporates the current, as well as earlier temperatures experienced by the organisms under study (for details, see Gienapp et al. 2005). A weighing factor α assesses the relative importance of current versus earlier temperatures: if this factor is large, the model places a high weight on the most recent temperature (the temperature variable resembles the mean temperature of that particular day), while if it is small, previous temperatures are given more importance, which implies a longer 'memory' of previous temperature conditions experienced by the organism. Values for the weighing factor ranged from 0.01 to 0.15 in 0.01 increments. In the model reduction process (based on a partial maximum likelihood method), we recalculated the best linear temperature predictor α in each reduction step. The mean December temperature of individual aviaries was used as a starting temperature value and daily mean temperatures modified by the weighing factor as time-dependent variables. Use of minimum temperatures did not give qualitatively different results. A similar approach was used to model the probability that a bird terminates reproduction. Statistical significance of variables in the proportional hazards model was tested with likelihood ratio tests (Therneau & Grambsch 2000). For the models including random effects we used the coxme and without random effects the coxph procedures in R 2.10.0 (R Development Core Team 2009).

Appendix 3.2 The effect of temperature on hormones

Assays

Plasma LH concentrations were determined using a chicken LH radioimmunoassay (Sharp et al. 1987) validated for use in blue tits (Caro et al. 2006), and plasma prolactin concentrations were determined using a recombinant derived starling prolactin radioimmunoassay (Bentley et al. 1997). Samples were assayed in duplicate, and in both assays the reaction volume was 60 µl comprising 20 µl of plasma sample or standard, 20 µl of primary rabbit LH or prolactin antibody, and 20 µl of ^{125}I-labelled LH or prolactin. The primary antibody was precipitated to separate free and bound ^{125}I label using 20 µl of donkey anti-rabbit precipitating serum and 20 µl of non-immune rabbit serum. All samples were measured in single assays. The intra-assay coefficient of variation for the LH and prolactin assays were 7.7 % and 6.5 %, and the minimum detectable doses were 0.08 ng/ml and 1.6 ng/ml, respectively.

Analysis

The LH and prolactin data were analyzed in a mixed model (procedure mixed in SAS 9.1, using Sattherwaite's method of calculating df) with sample period (date), treatment (warm/cold) and sex as fixed effects, and bird identity as a random effect.

Results

In 2006, in both males and females plasma LH concentrations were highest in January and February (Fig. S3.2) before the onset of gonadal development and were low during the egg laying period. We analyzed LH plasma concentrations weekly for 9 out of the 36 pairs (every bird was sampled once every 4 weeks) for 26 weeks (Fig. S3.2). There was no effect of the temperature treatment on LH. In a mixed model with bird identity as a random effect, sample date (as a factor with 25 levels) was highly significant ($F_{24,288}$=30.23, P<0.0001) but the main effects sex ($F_{1,69.4}$=0.41, P=0.52), treatment ($F_{1,69.1}$=1.41, P=0.24) and family ($F_{1,91.1}$=1.87, P=0.17) were not significant.

When we analyzed LH for each week separately (26 weeks), we found that temperature treatment affected LH concentrations in 3 weeks: 25th January ($F_{1,4}$=19.54, P=0.011, warm: 1.33, cold: 0.67 ng/ml LH), 23rd March ($F_{1,27}$=6.79, P=0.015, warm: 0.14, cold: 0.24 ng/ml LH) and 26th April ($F_{1,14}$=11.53, P=0.0044, warm: 0.18, cold: 0.32 ng/ml LH). Given the large number of tests and the relative small sample size per comparison we do not consider this strong support for a temperature effect on LH levels.

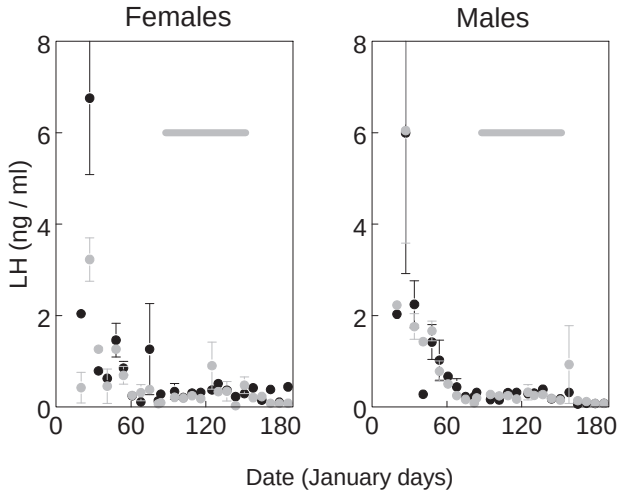

Figure S3.1: Luteinizing hormone (LH) concentrations (±SE) in male and female great tits kept at two temperature treatments in an aviary experiment in 2006. Every week 9 out of 36 pairs were sampled. The grey points represent the warm treatment, the black points the cold treatment. The grey horizontal bar indicates the range of the first egg dates. Date in January days (1=1st January).

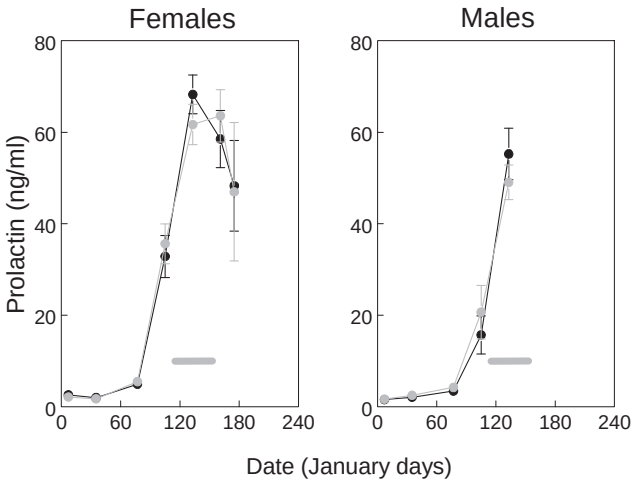

Figure S3.2: Prolactin concentrations (±SE) in male and female great tits in the same experiment. Grey points represent the warm treatment, black points the cold treatment. The grey horizontal bar indicates the range of the first egg dates. Date in January days (1=1st January).

In both sexes plasma prolactin increased in parallel with the increase in gonadal volume (Fig. S3.3). There was no effect of temperature treatment on prolactin plasma concentrations (Fig. S3.3), neither in interaction (sex*treatment: $F_{1,75.1}$=0.12, P=0.73; date*treatment: $F_{4,264}$=1.69, P=0.15) nor as main effect (treatment $F_{1,76}$=0.09, P=0.76). There were also no effects of family, neither in interaction with sex ($F_{7,54.8}$=1.69, P=0.13) nor as main effect ($F_{12,66.3}$=0.61, P=0.83). There were, however, differences between males and females ($F_{1,75.4}$=16.7, P=0.0001) and in both sexes prolactin concentration changed over time. When analyzed per sample period all P-values comparing the treatments were >0.17.

Discussion

The seasonal patterns of LH were similar in males and females, with a single and brief peak occurring in January after which the levels remained low in both temperature treatments. This unexpected seasonal profile was also observed in other studies on great tits held in captivity in which the rapid increase in LH was associated with the transfer of the birds from short to long day length (Silverin & Viebke 1994; Silverin et al. 2008), reflecting a very rapid integration of photoperiodic cues (Meddle & Follett 1997; Saab et al. 2010). In our experiment however, birds were not exposed to a sudden increase in photoperiod, ruling out this potential photoperiodic induction effect. Why LH rose so quickly at the beginning of the pre-breeding season when photoperiod is still short remains unclear. It is unlikely that this is artefact of captivity as in other years the LH pattern did peak just prior to breeding (Schaper et al. 2012). Another open question is how birds can grow their gonads and breed with very low amounts of LH, although high circulating levels of hormones are not always necessary for maturating the reproductive machinery (Tramontin et al. 2001; Caro et al. 2005b) as long as plasma carriers and/or hormones receptors are present in sufficient numbers (Ball & Balthazart 2008).

There was no effect of temperature on the time or rate of increase in prolactin, which is consistent with an earlier study on starlings (Dawson & Sharp 2010). However, there is evidence from starlings that the start of moult is related to the time of peak prolactin concentrations (Dawson 2006; Dawson & Sharp 2010). Although great tits in this study started to moult sooner at the higher temperatures, prolactin in females appeared to peak sooner in birds at the lower temperature. However, the resolution of changes in prolactin concentrations (blood samples taken once every four weeks) was insufficient to be certain of this. Blood samples were not taken from males long enough to determine the time of peak values.

Effects of temperature on gonadal regression (see main text) and moult (see Appendix 3.4) has been reported in most species in which it has been investigated (Silverin & Viebke 1994; Wingfield et al. 1997; Wingfield et al. 2003; Dawson 2005a; Silverin et al. 2008), and contrasts with the effect of temperature on the onset of breeding that produced more ambiguous results. This suggests that the mechanisms translating temperature at the neuro-endocrine level differ between the onset and the termination of reproduction. One possible explanation for the early termination of breeding under warm temperature involves prolactin secretion. In many bird species, peaks of prolactin concentrations coincide with the onset of moult, and therefore with the termination of reproduction (e.g. Sharp et al. 1998; Dawson 2005a).

Data from turkeys (*Meleagris gallopavo*, Gahali et al. 2001) and male, but not female, white-crowned sparrow (*Zonotrichia leucophrys oriantha*, Maney et al. 1999) suggests that warmer temperatures may lead to higher prolactin concentrations in the blood. However, high temperature had no effect on photoinduced prolactin secretion in either sex of *Z.l. gambelli* or *Z.l.pugetensis* (Maney et al. 1999) nor in male song sparrows (*Melospiza melodia morphna*, Perfito et al. 2005). Dawson and Sharp (2010) found that although higher temperature did not enhance prolactin concentrations in starlings, it did advance the time that prolactin concentrations began to decline. Consequently, the seasonal peak in prolactin concentrations was advanced, and this correlated with the advance in the start of moult. Our data also show that temperature had no effect on the time or magnitude of the increase in prolactin concentrations. In females there was no effect on the time of peak prolactin concentrations (this could not be assessed in males). While it has been recently shown that prolactin and gonadal regression are not necessarily tightly associated (Dawson 2006), the absence of a correlation between prolactin and onset of moult is more surprising and remains unexplained at present.

Appendix 3.3 The effect of temperature on moult

Introduction

Temperature may constitute a meaningful cue to initiate egg laying but it may also play a role in the termination of reproduction which is, like the initiation of the breeding cycle, coupled to a restructuring of body tissues and culminates in the onset of the postnuptial moult. In this context it is important to point out that a later onset of moult will likely lead to a faster speed of moult. The speed of moult affects the structure and functionality of the moulted feathers and therefore influences the bird's condition in the next breeding season (Dawson et al. 2000; de la Hera et al. 2009). Dawson (2005b) showed that male starlings kept in climate controlled indoor aviaries started moulting earlier when kept at higher room temperatures.

Methods

Moult of each of the primary wing feathers was scored once every four weeks from the end of breeding onwards as 0 (no new feather), 0.25, 0.5, 0.75 or 1 (complete new feather). From this a moult score was calculated for each of the birds following Dawson & Newton (2004), using the great tit specific parameters from Dawson (2005b). For each individual, date was linearly regressed against moult score (for all scores > 0) and from this the onset of moult (intercept) and speed of moult (slope) were determined.

The moult data (onset and speed) were analyzed in a mixed model (procedure mixed in SAS 9.1, using Sattherwaite's method of calculating df) with treatment (warm/cold) and sex and their interaction as fixed effects, and family as a random effect. In a separate generalized linear model (procedure glm in SAS 9.1) we tested for family differences (with family as a fixed effect) in the onset of moult.

Results

Similar to the termination of reproduction (see main text), the onset of moult was clearly affected by temperature treatment: birds in the warmer treatment started moult earlier (Table S3.1 & Fig. S3.4). There were additional effects of year (earlier onset in 2007), laying date (birds that laid later also moulted later) and the date of the last egg in interaction with sex: the onset of moult in females was much more affected by the date of her last egg (0.60 days later per day of last egg) than males (0.14 days later per day). There was no additional effect of family ($F_{20,93}$=1.53, P=0.09).

The duration of moult was strongly affected by the onset of moult (Table S3.1 & Fig. S3.4): for every day a bird started moulting later, the duration of moult decreased with half a day. On top of this effect, females, which also started later, moulted on average 6 days faster.

Table S3.1: The onset (in January days, 1=1st January) and duration (days) of moult of male and female great tits kept at two temperature treatments in an experiment replicated over two years. Pairs differed in their onset of reproduction (laying date) and their termination of reproduction (last egg date).

Explanatory variable	Estimate	SE	F	P
Onset				
Year 2006	57.37	5.67	$F_{1,112}$=12.14	**0.0007**
Year 2007	51.35	5.97		
Treatment: cold	4.19	1.57	$F_{1,112}$=7.12	**0.0088**
Treatment: warm	0			
Sex: female	-17.93	7.36	$F_{1,112}$=5.94	**0.016**
Sex: male	0			
Laying date	0.20	0.068	$F_{1,112}$=8.32	**0.0047**
Last egg date*Sex female	0.61	0.076	$F_{2,112}$=32.86	**<0.0001**
Last egg date*Sex male	0.14	0.078		
Duration				
Onset of moult	-0.53	0.050	$F_{1,135}$=110.84	**<0.0001**
Sex: female	105.6	4.33	$F_{1,135}$=574.78	**<0.0001**
Sex: male	111.6	3.71		

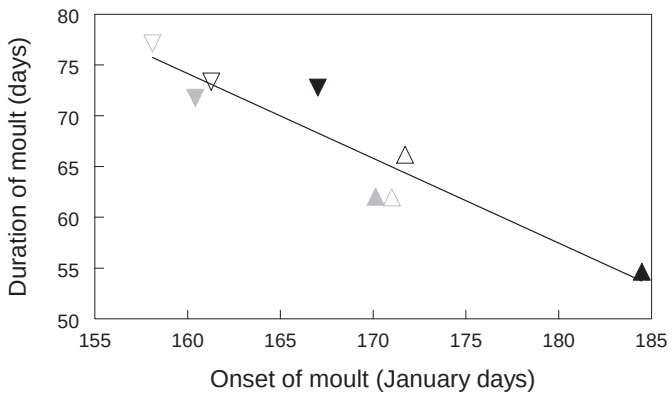

Figure S3.3: The duration (days) versus the onset (January days, 1=1st January) of moult of male and female great tits kept at two temperature treatments in an experiment replicated over two years. The black symbols represent the cold treatment, the grey symbols he warm treatment, the closed symbols represent 2006, the open symbols 2007, the down-facing triangle represent females, the up-facing triangle males.

Appendix 3.4: Temperature treatments

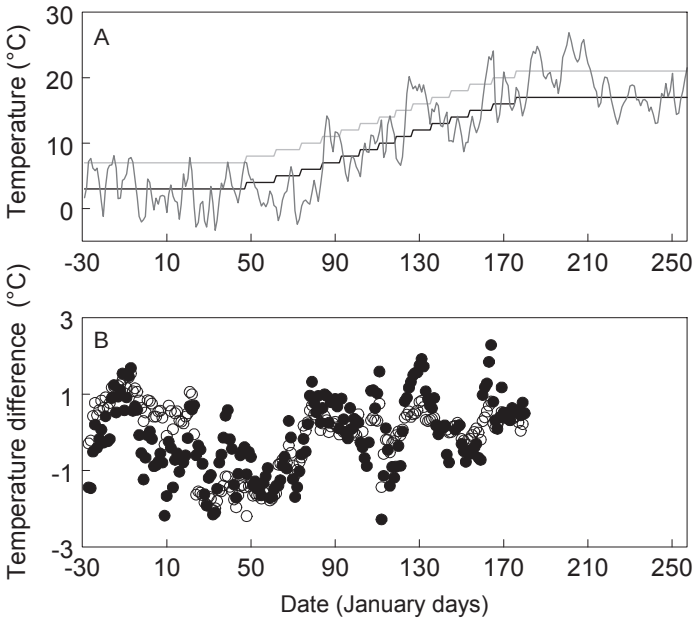

Figure S3.4 (**A**) The target temperatures for the two temperature treatments at which pairs of great tits were kept in a two year experiment. The black line represents the cold treatment, the light grey line the warm treatment. The average outside temperatures (dark grey line) for the Netherlands (De Bilt) are also indicated. (**B**) The difference in daily realized temperatures in the aviaries in 2006 versus 2007. The closed symbols are the cold treatment; the open symbols the warm treatment.

Appendix to chapter 4

Appendix 4.1: Termination of Reproduction

We calculated temperature effects on the timing of the termination of reproduction with Cox proportional hazards models. In 2008, late-born females stopped laying later than early-born females after experiencing a cold period in March, whereas they stopped earlier than early-born females when they started laying under cold conditions in April (Table S4.1a; Figs. S4.1, S4.2). In a model including realized temperatures, the best weighting factor was very high, indicating that the decision to stop laying was based on most recently experienced temperatures (Table S4.1b). Thus, after a cold temperature spell, late-born females were more likely to terminate reproduction early than early-born females. In 2009, neither treatment nor realized temperature influenced the termination of reproduction (Table S4.1). In 2010, late-born females terminated reproduction earlier than early-born females after a late temperature rise in late spring, whereas the laying dates were similar if the temperature rise happened two weeks earlier in late spring (Table S4.1a; Figs. S4.1, S4.3). There was no effect of realized temperature on the termination date (Table S4.1b). We found a consistent resemblance between sisters in the termination of laying in all years (female family: 2008: χ^2_{10}=30.25, P<0.001; 2009: χ^2_{14}=29.15, P=0.010; 2010: χ^2_{8}=16.52, P=0.036), possibly showing genetic differences in temperature sensitivity between early- and late-laying families, as these varied in termination dates in response to temperature.

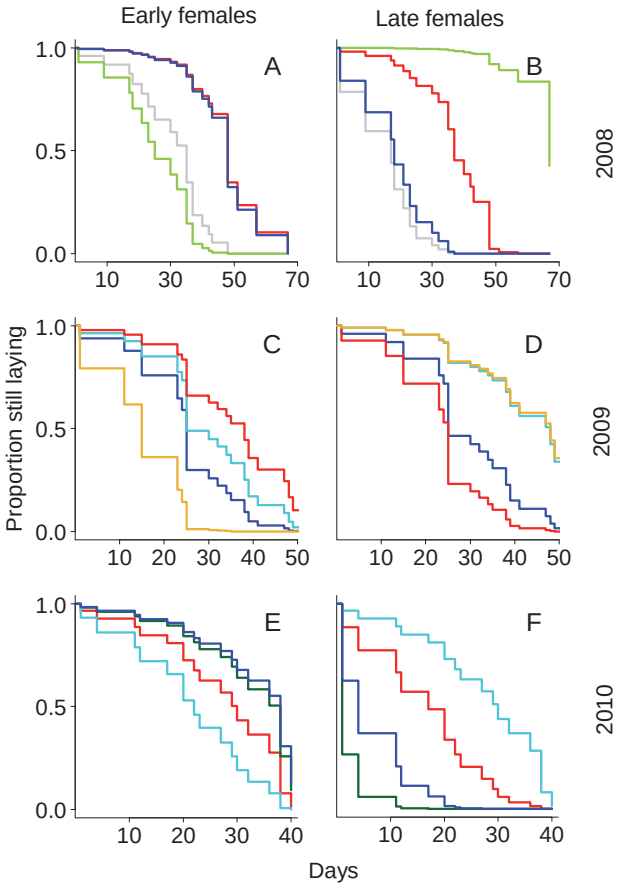

Figure S4.1: Effect of temperature treatments on the termination of laying for females from early (left) and late (right) families in climate-controlled aviaries in 2008 (A, B), 2009 (C, D), and 2010 (E, F): survival graphs showing the outcome of the proportional hazards model in Table S4.1a. A, C, and E show the earliest-laying family of each year (mother's laying dates: 7th April, 2008; 3rd April, 2009; 8th April, 2010) and B, D, and F the latest-laying family (mother's laying dates: 20th April, 2008; 26th April, 2009; 18th April, 2010). Days represent days after the first termination date: 5th May, 2008, 1st May, 2009, and 29th April, 2010. A fast-descending line represents a group that stops laying early. Colours correspond to the treatments shown in Figure 4.1.

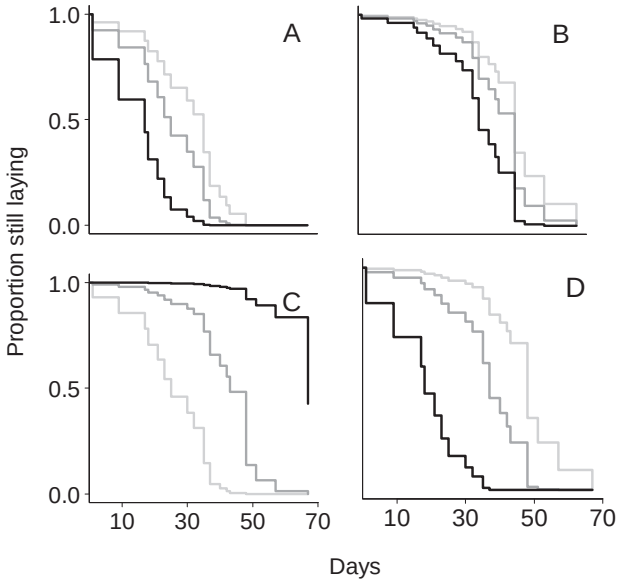

Figure S4.2: Termination of laying in 2008: survival graphs showing the outcome of the proportional hazards model in Table S4.1a. Each panel depicts one of the four temperature treatments: A, constant temperature of 15°C; B-D, cold period in February, March, or April, respectively. Lines show the proportions of females that were still laying from the families with the earliest (7th April, light grey), mean (12th April, grey), and latest (20th April, black) laying dates. Days represent days after the first termination date, 5th May.

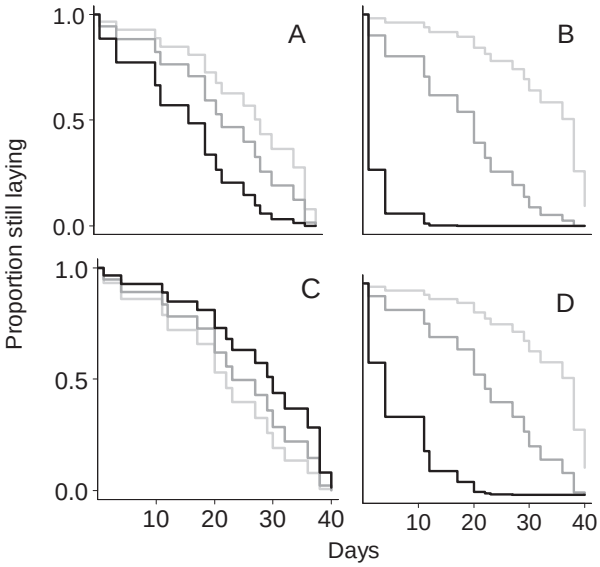

Figure S4.3: Termination of laying in 2010: survival graphs showing the outcome of the proportional hazards model in Table S4.1 using temperature treatment and individual characteristics. Each panel depicts one of the four temperature treatments: A, fast advancing early spring, early late spring; B, fast-advancing early spring, late late spring; C, slowly advancing early spring, early late spring; D, slowly advancing early spring, late late spring. Lines show the proportion of females that were still laying from the families with the earliest (8th April, light grey), mean (12th April, grey), and latest (18th April, black) laying dates. Days represent days after the first termination date, 29th April.

Table S4.1: Effects of temperature treatment, or realized temperature, and individual traits on the termination of reproduction. We tested how the termination of reproduction was affected by temperature treatment (a) or realized temperature (b) and individual traits in the years 2008-2010. The best weighting factor a is given in the "df" column for each year. Female family was fitted as a random effect. Statistics are given for the point of exclusion from the model. In case of significant interactions, statistics for the components are given in the presence of the interaction. Therefore, statistics for a continuous variable cannot be provided for an interaction of this variable and a factor.

	2008			2009			2010		
	χ^2	df	P	χ^2	df	P	χ^2	df	P
a) Temperature treatment and individual traits									
Temperature treatment	9.67	3	**0.022**	6.58	3	0.087	12.90	3	**0.005**
Laying date of female's mother				0.06	1	0.80			
Treatment*Laying date of female's mother	13.30	3	**<0.001**	2.12	3	0.15	14.00	3	**<0.001**
Laying date of male's mother	1.62	1	0.23	0.32	1	0.58	6.36	1	**0.016**
Female personality	0.09	1	0.77	2.84	1	0.080	1.08	1	0.31
Female chick weight	0.63	1	0.43	0.14	1	0.71	8.25	1	**0.018**
Male personality	2.32	1	0.13	1.79	1	0.14	11.50	1	**0.003**
Male chick weight	0.17	1	0.68	0.35	1	0.56	0.04	1	0.97
Pair composition				3.72	3	0.29			
b) Realized temperature and individual traits									
Realized temperature	2.58	1	0.11	0.01	1	0.92	0.05	1	0.84
Laying date of female's mother	7.31	1	**0.007**	0.07	1	0.79	1.44	1	0.24
Temperature*Laying date of female's mother	5.54	1	**0.025**	0.18	1	0.67	<0.01	1	0.96
Laying date of male's mother	0.03	1	0.86	0.05	1	0.80	0.51	1	0.44
Female personality	0.17	1	0.68	2.84	1	0.085	1.21	1	0.29
Female chick weight	0.05	1	0.79	0.13	1	0.72	0.51	1	0.51
Male personality	1.63	1	0.22	1.79	1	0.14	0.30	1	0.60
Male chick weight	6.74	1	**0.028**	<0.001	1	1	1.17	1	0.27
Pair composition				4.63	1	0.20			
Best weighing factor α		0.2			0.11			0.01	

Appendix 4.2: Onset and Duration of Molt

Molt of the primary wing feathers was recorded biweekly in 2008 and weekly in 2009 and 2010. The molt score was calculated following Dawson and Newton (2004), with great tit-specific parameters from Dawson (2005b). For each individual, date was linearly regressed against molt score. The onset (intercept) and speed (slope) of molt were used to calculate molt duration. In most years, nonlaying birds started molting significantly earlier than laying pairs. In general, the later a bird stopped reproducing, the later it started molting. There was no effect of temperature treatments on the onset of molt. Some birds experiencing a cold spring period in 2008 molted significantly faster, even though molt took place under constant warm conditions.

Table S4.2: Effects of temperature treatment and individual traits on the onset and duration of moult. Onset and duration of moult in 2008-2010 were analyzed in mixed models using family as a random effect. First, laying and non-laying birds were compared, showing that birds that skip reproduction mostly start moulting earlier than those that do not. A subsequent analysis included only laying pairs and used date of termination of reproduction, temperature treatment, and date of birth (laying date of the mother) as fixed effects. Statistics are given for the point of exclusion from the model.

	2008			2009			2010		
	χ^2	df	P	χ^2	df	P	χ^2	df	P
Females									
a) Onset of moult									
Laying - non-laying	17.79	1	**<0.001**	6.01	1	**0.014**	0.09	1	0.77
Temperature treatment	6.27	3	0.099	2.53	3	0.47	0.49	3	0.92
Laying date last egg	21.67	1	**<0.001**	14.00	1	**<0.001**	14.60	1	**<0.001**
Laying date of mother	0.02	1	0.88	1.93	1	0.17	0.18	1	0.67
b) Duration of moult									
Laying - non-laying	8.52	1	**0.004**	0.50	1	0.48	0.03	1	0.86
Temperature treatment	16.36	3	**<0.001**	4.64	3	0.20	2.90	3	0.40
Laying date last egg	<0.01	1	1	0.12	1	0.73	<0.01	1	0.98
Laying date of mother	1.09	1	0.30	<0.01	1	0.96	0.15	1	0.70
Males									
a) Onset of moult									
Laying - non-laying	1.56	1	0.21	4.73	1	**0.030**	0.17	1	0.68
Temperature treatment	6.91	3	0.075	6.72	3	0.081	4.63	3	0.20
Laying date last egg	0.37	1	0.54	9.05	1	**0.003**	6.27	1	**0.012**
Laying date of mother	0.13	1	0.72	1.02	1	0.31	<0.01	1	0.97
b) Duration of moult									
Laying - non-laying	0.41	1	0.52	0.02	1	0.90	0.57	1	0.45
Temperature treatment	11.17	3	**0.011**	7.23	3	0.065	2.74	3	0.43
Laying date last egg	0.23	1	0.63	0.66	1	0.42	1.86	1	0.17
Laying date of mother	1.81	1	0.18	<0.01	1	1	0.91	1	0.34

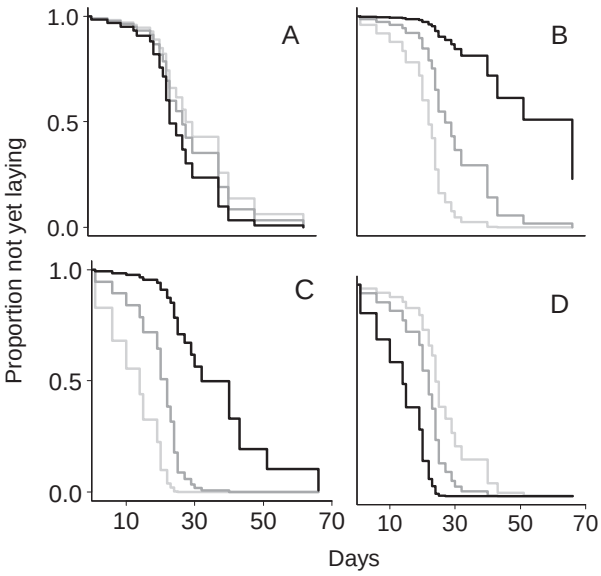

Figure S4.4: Survival graphs showing the outcome of the proportional hazards model in Table 4.1a. Each panel depicts one of the four temperature treatments: A, constant temperature of 15°C; B-D, cold period in February, March, or April, respectively. Lines show the proportion of females that had not yet started laying at the given date for the families with the earliest (7th April, light grey), mean (12th April, grey), and latest (20th April, black) laying dates. Days represent days after the first laying date, 17th April. In 2008, cold conditions in February or March, followed by a temperature rise, made early-laid females lay earlier compared to early-laid females in constant warm conditions and early-laid females that experienced a cold April with no subsequent temperature rise. In contrast, cold conditions in February/March made late-laid females lay significantly later.

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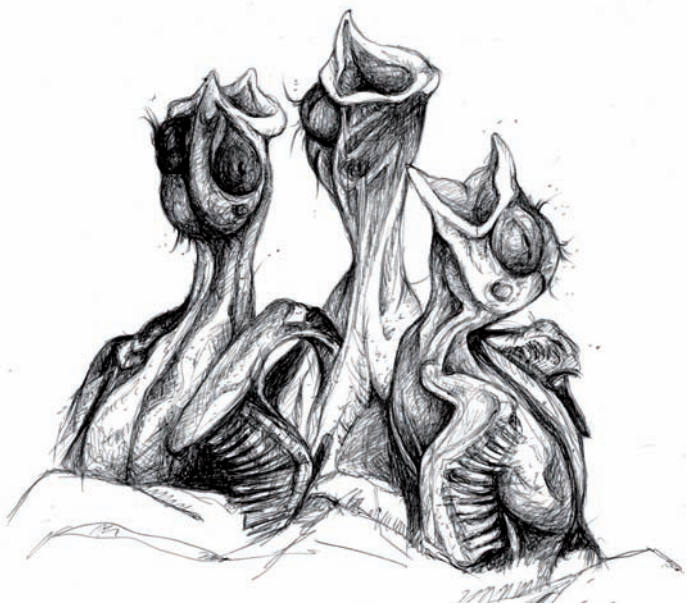

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Summary

Understanding how species adapt to a changing environment is central to ecology and evolutionary biology. Energetically intense life-history stages, such as reproduction, have to be matched to favourable environmental conditions to maximize fitness benefits. As the optimal time for these events varies from year to year, organisms are adapted to fine-tune their reproductive timing to the seasonal phenology of their environment.

In temperate zone woodlands, a warm spring induces earlier bud burst of trees and the earlier emergence of caterpillars that form the principal food source for great tit (*Parus major*) nestlings. To anticipate the period when sufficient food is available for their young and thus the optimal time to reproduce, females use environmental cues to determine when to start egg laying. Besides the increasing photoperiod, temperature is thought to be the most influential predictive cue for the birds, which have to start breeding earlier in a warm year to match their chick-rearing phase with the seasonal food peak.

Due to climate change, spring temperatures have increased in central Europe and as a consequence the bud burst of trees and emergence of caterpillars that forage on the young leaves have advanced substantially. The period in which great tit females lay their eggs has also advanced, but not sufficiently to keep track of this change. Consequently, selection for early laying has intensified in recent years, but a large variation in the onset of laying is still present, with many females laying later than would be optimal to raise a brood fledging in good condition. The physiological basis for this variation in laying dates is not well understood. According to the 'cues hypothesis', females differ in the sensitivity to predictive environmental cues, and thus only some females are able to interpret the changing temperature cues adequately and advance laying accordingly. In contrast, according to the 'constraints' hypothesis, early breeding comes with a cost, as eggs have to be produced in colder conditions, and thus differences in energetic capabilities explain why some females breed later than would be optimal for their chicks.

The aim of this thesis was to investigate proximate mechanisms underlying the seasonal timing of reproduction in great tits, with a focus on the use of temperature cues. Additionally, the potential for micro-evolutionary adaptation to long-term directional climate change was examined.

Phenotypic plasticity is the ability to change a phenotypic trait in response to different environments. Plasticity in the onset of egg laying in response to temperature is usually demonstrated by regressing the mean laying date of a bird population against the mean temperature in a fixed period. This relationship was more closely investigated with an individual-based approach using pairs of great tits breeding in climate-controlled aviaries under different temperature scenarios. In this case, however, mean temperature was not the relevant cue determining the onset of egg laying, but rather

the pattern of temperature increase better explained variation in laying dates. The use of genetically related females made it possible to identify a family resemblance in the onset and termination of egg laying in response to temperature cues, which indicates a genetic basis. Individual variation in the onset of laying in response to a shared environment in a given year is thus partly determined by different sensitivities to temperature patterns. The heritability of phenotypic plasticity determined in wild birds could therefore be the result of heritable cue sensitivity. This heritability would facilitate micro-evolutionary changes in the timing of reproduction in response to a warming climate which would allow great tits to eventually re-establish the synchrony of their breeding cycle with their seasonal environment. Other theories about the ways in which temperature could cause variation in the onset of egg laying, such as microclimatic temperature differences between individual breeding territories or indirect effects via vegetation phenology or presence of food items, could not be supported.

Prior to egg laying, birds have to mature their ovaries or testes from their regressed winter state, a slow process that is stimulated by the seasonal increase in photoperiod. An investigation of the pre-laying changes in reproductive hormone titers and gonadal growth under controlled conditions showed that moderate differences in mean temperature or temperature patterns did not affect the timing of these physiological changes. This lack of a temperature-related regulation could at some point potentially restrict a further advancement of egg laying in a warmer climate. Since the timing of ovarian follicle maturation was partly genetically determined, an adaptation by earlier gonadal maturation is likely possible. However, females showed a remarkable plasticity in the time they laid their first egg independent of the state of gonadal development in late spring. This implies that physiological constraints are currently not responsible for the variation in laying dates found under natural conditions. This was also confirmed by the fact that photostimulation of wild great tit females in spring, which was expected to accelerate gonadal growth, did not lead to earlier egg laying.

In nature, great tit egg size varies between females, but also within the same clutch of eggs. Slight seasonal increases in egg size are observed in the wild, in concert with an increase in mean temperature, but also prey availability. In contrast, under controlled conditions and ad libitum food slightly larger eggs were laid under colder temperatures. It is therefore more likely that the seasonal increase in egg size that is a common pattern in small song birds is driven by food availability and possibly indirect temperature effects on the energy metabolism.

Predicting the effect of future climate change on the population viability of small song birds is a difficult task, especially taking the proposed influence of rising temperatures on seasonal timing of reproduction into account. The major environmental component that drives the seasonal phenology of the oak-winter moth-great tit food chain is ambient temperature. However, different temperatures affect various components of this food chain at different times in spring. Within each species, it is also important to consider that linked life-cycle stages are to a higher or lower degree fine-tuned to the seasonal environment by temperature cues: while the onset of egg laying in great tits seems to be determined by patterns of temperature change, this was not the case for the underlying reproductive physiology or the onset of moult in late summer. A disruption of phenological patterns is thus likely. Investigating the physiological basis of seasonal timing in a changing climate can help us understand shifts in population and food web dynamics, which can potentially affect species abundances and community composition.

Summary

Samenvatting

De vraag hoe soorten zich aanpassen aan een veranderende omgeving staat centraal in de ecologie en de evolutiebiologie. Levensfasen die veel energie kosten, zoals voortplanting, moeten worden gesynchroniseerd met gunstige omgevingsfactoren om fitness te maximaliseren. Aangezien het optimale tijdstip van deze gebeurtenissen varieert van jaar tot jaar, zijn organismen in staat om de timing van voorplanting af te stemmen op de fenologie van hun omgeving.

In gematigde bosgebieden leidt een warm voorjaar tot het eerder ontstaan van knoppen aan de bomen en het eerder verschijnen van rupsen die de belangrijkste voedselbron vormen voor nestjongen van de koolmees (*Parus major*). Om te anticiperen op de periode wanneer er voldoende voedsel beschikbaar is voor hun jongen en dus de ideale tijd om te reproduceren, gebruiken vrouwtjes omgevingsfactoren om te bepalen wanneer met de eileg te beginnen. Naast de toenemende daglengte, wordt temperatuur gezien als het belangrijkste signaal voor vogels, die in een warm jaar eerder moeten beginnen met voortplanten om de kuikenfase af te stemmen op de piek in het voedselaanbod.

Als gevolg van klimaatverandering zijn de voorjaarstemperaturen in Midden-Europa gestegen met als gevolg dat het uitlopen van bomen en de ontwikkeling van rupsen die de jonge bladeren eten aanzienlijk is vervroegd. De periode waarin koolmees vrouwtjes hun eieren leggen is ook vervroegd, maar niet voldoende om deze verandering bij te houden. Daardoor is selectie voor het eerder leggen van eieren in de afgelopen jaren versterkt. Er is echter nog steeds een grote variatie in de aanvang van eileg aanwezig, met veel vrouwtjes die later eieren leggen dan wat optimaal zou zijn om hun kroost in goede conditie groot te brengen. De fysiologische basis voor deze variatie in legdatum is nog onvoldoende begrepen. Volgens de 'signalen hypothese' verschillen vrouwtjes in de gevoeligheid om omgevingsfactoren te voorspellen en zijn dus slechts enkele vrouwtjes in staat om adequaat veranderende temperatuursignalen te interpreteren en dienovereenkomstig het leggen van eieren te vervroegen. Volgens de 'beperkingen hypothese' heeft vroeg broeden een prijs aangezien eieren geproduceerd moeten worden in koudere omstandigheden en dus verschillen in energetische mogelijkheden verklaren waarom sommige vrouwtjes later broeden dan optimaal zou zijn voor haar jongen.

In dit proefschrift worden de proximate mechanismen die ten grondslag liggen aan de timing van voortplanting in koolmezen onderzocht, met een focus op het gebruik van temperatuursignalen. Bovendien was de mogelijke micro-evolutionaire aanpassing aan langdurige klimaatsverandering onderzocht.

Fenotypische plasticiteit is de mogelijkheid om een eigenschap te veranderen als reactie op verschillende milieus. Plasticiteit in de start van de eileg in reactie op temperatuur wordt meestal aangetoond door de gemiddelde legdatum van een vogelpopulatie uit te zetten tegen de gemiddelde temperatuur in een bepaalde periode.

Deze relatie is nader onderzocht op basis van een individuele aanpak waarbij paartjes koolmezen broedden in geklimatiseerde volières onder verschillende temperatuur scenario's. Echter in dit geval bleek niet de gemiddelde temperatuur het meest relevante signaal te zijn die de start van eileg bepaalde, maar veeleer het patroon van temperatuurstijging dat de variatie in legdatum verklaarde. Het gebruik van genetisch verwante vrouwtjes maakte het mogelijk om familiegelijkenissen te herkennen bij de start en het eind van de eileg in reactie op temperatuursignalen, wat een genetische basis aangeeft. Individuele variatie in de start van eileg, ontstaan door het delen van een omgeving in een bepaald jaar, wordt dus mede bepaald door een verschillende gevoeligheid voor temperatuurpatronen. De erfelijkheid van fenotypische plasticiteit in wilde vogels zou dus het gevolg kunnen zijn van erfelijke verschillen in gevoeligheid voor temperatuursignalen. Deze erfelijkheid zou micro-evolutionaire veranderingen in de timing van voortplanting in reactie op een opwarmend klimaat mogelijk maken, wat ervoor zal zorgen dat koolmezen uiteindelijk de synchronie van hun broedcyclus kunnen herstellen. Er werd geen steun gevonden voor andere theorieën hoe temperatuur kan leiden tot variatie in de start van eileg, zoals micro-klimatologische temperatuurverschillen tussen individuele territoria, indirecte effecten via vegetatie fenologie of de aanwezigheid van voedsel.

Voorafgaand aan de eileg moeten vogels hun eierstokken of testikels laten rijpen van hun regressieve winter toestand, een langzaam proces wat gestimuleerd wordt door de seizoensgebonden toename van de daglengte. Onderzoek onder gecontroleerde omstandigheden naar veranderingen in concentraties van reproductiehormonen en gonadale groei in de periode voor de eileg laten zien dat de matige verschillen in de gemiddelde temperatuur of temperatuurpatronen niet van invloed zijn op de timing van deze fysiologische veranderingen. Het ontbreken van een temperatuur-gerelateerde regulatie zou op een gegeven moment de verdere ontwikkeling van de eileg in een warmer klimaat kunnen beperken. Aangezien de timing van ovariële follikelrijping gedeeltelijk genetisch was bepaald, is een aanpassing van eerdere gonadale rijping waarschijnlijk mogelijk. Echter, in het late voorjaar toonden vrouwtjes een opmerkelijke plasticiteit in het tijdstip dat zij hun eerste ei legden onafhankelijk van de ontwikkelingstoestand van hun gonaden. Dit houdt in dat fysiologische beperkingen momenteel niet verantwoordelijk zijn voor de variatie in legdatum zoals gevonden onder natuurlijke omstandigheden. Dit werd ook bevestigd door het feit dat fotostimulatie van wilde koolmeesvrouwtjes in het voorjaar, waarvan verwacht werd dat deze de gonadale groei zou versnellen, niet hebben geleid tot het eerder leggen van eieren.

In de natuur varieert de grootte van koolmeeseieren tussen vrouwtjes, maar ook binnen hetzelfde broedsel. Na verloop van tijd neemt eigrootte geleidelijk toe in het voorjaar, gezamenlijk met een toename van de gemiddelde temperatuur en een toename van voedselbeschikbaarheid. Dit in tegenstelling tot gecontroleerde

omstandigheden met onbeperkte voedselbeschikbaarheid, waar iets grotere eieren werden gelegd bij koudere temperaturen. Het is dan ook waarschijnlijker dat de seizoensgebonden toename in eigrootte, die in kleine zangvogels vaak werd gevonden, wordt veroorzaakt door beschikbaarheid van voedsel en mogelijk indirecte temperatuureffecten op de energiehuishouding.

Het voorspellen van effecten van toekomstige klimaatverandering op de populatiedynamica van kleine zangvogels is moeilijk, in het bijzonder wanneer de mogelijke invloed van stijgende temperaturen op de timing van voortplanting er bij wordt betrokken. De belangrijkste omgevings-component die de seizoensgebonden fenologie van de eik-wintervlinder-koolmees voedselketen aandrijft is de omgevingstemperatuur. Echter, verschillende temperaturen beïnvloeden diverse onderdelen van dit voedselketen op verschillende momenten in het voorjaar. Zelfs gerelateerde fasen in de levenscyclus van vogels zijn in bepaalde mate afgestemd op het seizoensgebonden milieu door temperatuursignalen: terwijl de start van de eileg lijkt te worden bepaald door de patronen van temperatuurverandering bleek dit niet het geval te zijn voor de onderliggende reproductieve fysiologie, of het begin van de rui in de nazomer. Een verstoring van de fenologische patronen is dus waarschijnlijk. Onderzoek naar de fysiologische basis van seizoenstiming in een veranderend klimaat kan ons verschuivingen in de dynamica van voedselwebben helpen begrijpen, die op hun beurt weer veranderingen in de (kwantitatieve) samenstelling van levensgemeenschappen meebepalen.

Zusammenfassung

Es ist ein zentrales Thema der Ökologie und Evolutionsbiologie zu begreifen wie sich Arten an eine verändernde Umwelt anpassen. Aus energetischer Sicht aufwendige Life-history-Stadien, wie die Fortpflanzung, müssen mit günstigen Umweltbedingungen abgestimmt werden um den Fitness-Gewinn zu maximieren. Da jedoch der optimale Zeitpunkt für diese Ereignisse von Jahr zu Jahr variiert, sind Organismen daran angepasst ihre Fortpflanzung an die Phänologie der Umwelt anzugleichen.

In Laubwäldern der gemäßigten Breiten bewirkt ein warmes Frühjahr einen früheren Blattaustrieb der Bäume und das frühere Auftreten von Raupen, die die Hauptnahrung für Kohlmeisennestlinge darstellen. Für eine erfolgreiche Fortpflanzung müssen die Weibchen den optimalen Zeitraum für die Fortpflanzung voraussehen, in dem ausreichend Nahrung für die Jungen zur Verfügung steht. Sie benutzen Signale aus ihrer Umwelt, die ihnen ermöglichen die Zeit für den besten Legebeginn zu bestimmen. Neben der ansteigenden Tageslänge wird in diesem Zusammenhang Temperatur als wichtigstes Signal für die Vögel angesehen, die in einem warmen Jahr früher mit dem Brüten anfangen müssen um die Jungenaufzucht mit dem saisonalen Nahrungsmaximum zu synchronisieren.

In Verbindung mit dem Klimawandel sind die Frühjahrstemperaturen in Mitteleuropa gestiegen und als Folge finden auch der Blattaustrieb der Bäume und das Auftreten der Raupennahrung verfrüht statt. Der Zeitraum, in dem Kohlmeisen ihre Eier legen, hat sich ebenfalls vorverlagert, doch nicht genug um diesem Wandel zu folgen. Daher hat sich die Selektion für einen frühen Legebeginn in den letzten Jahren verstärkt. Trotzdem ist noch stets Variation im Legezeitpunkt vorhanden, so dass viele Weibchen später Eier legen als es optimal wäre um eine Brut aufzuziehen, die beim Ausfliegen in guter Verfassung ist. Die zugrundeliegende physiologische Basis dieser Variation ist bisher nicht bekannt. Nach der Signal-Hypothese (cues hypothesis) unterscheiden sich Weibchen in ihrer Sensitivität für Umweltsignale mit hohem Vorhersagewert, so dass nur manche Weibchen die veränderten Temperatur-Signale richtig deuten und ihr Legedatum vorverlegen. Im Gegensatz dazu besagt die Beschränkungs-Hypothese (constraints hypothesis) dass frühes Eierlegen mit Kosten verbunden ist, weil Eier unter kälteren Bedingungen produziert werden müssen und daher Unterschiede im energetischen Vermögen erklären warum manche Weibchen späten brüten als es für ihre Jungen optimal wäre.

Die Zielsetzung dieser Dissertation war es, proximate Mechanismen zu erklären, die dem saisonalen Timing der Fortpflanzung von Kohlmeisen zu Grunde liegen, wobei der Schwerpunkt auf der Verwendung von Temperatursignalen lag. Darüber hinaus wurde das Potential für mikro-evolutionäre Anpassungen an den langzeitigen Klimawandel erforscht.

Phänotypische Plastizität ist die Fähigkeit ein phänotypisches Merkmal als Reaktion auf unterschiedliche Umweltbedingungen zu verändern. Plastizität im Legebeginn von Vögeln wird gewöhnlich demonstriert, indem das Durchschnittslegedatum einer Vogelpopulation mit der Durchschnittstemperatur eines festgelegten Zeitraumes korreliert wird. Dieses Verhältnis wurde mit Hilfe eines individuenbasierten Ansatzes näher untersucht, wobei Kohlmeisenpaare in klimatisierten Volieren unter verschiedenen Temperaturszenarien brüteten. Hierbei stellte die Durchschnittstemperatur jedoch keinen relevanten Stimulus dar um den Legebeginn festzulegen, stattdessen erklärte das Muster des Temperaturanstiegs die Variation im Legedatum. Der Einsatz genetisch verwandter Weibchen machte es möglich die Gemeinsamkeiten der Familien im Timing des Beginns und des Endes der Legeperiode in Reaktion auf Temperatursignale festzustellen. Dieses Resultat deutet auf eine genetische Basis hin. Individuelle Variation im Legebeginn als Reaktion auf die geteilte Umgebung ist demzufolge teilweise durch Unterschiede in der Sensitivität gegenüber Temperaturmustern zu erklären. Die Erblichkeit phänotypischer Plastizität, die in wilden Vögeln festgestellt wurde, könnte also das Resultat erblicher Sensitivität gegenüber Umweltsignalen sein. Diese Erblichkeit würde mikro-evolutionäre Veränderungen im Timing der Fortpflanzung als Reaktion auf die Klimaerwärmung ermöglichen, was Kohlmeisen letztendlich erlauben würde, ihren Brutzyklus wieder mit der saisonalen Umwelt in Einklang zu bringen. Andere Theorien über die Art und Weise in der Temperaturunterschiede die Variation im Legebeginn verursachen könnten, zum Beispiel über mikroklimatische Temperaturunterschiede zwischen den einzelnen Brutterritorien oder indirekte Einflüsse über die Phänologie der Vegetation oder das Auftreten von Nahrung, konnten nicht bestätigt werden.

Vor dem Eierlegen müssen Vögel ihre Ovarien oder Hoden aus dem zurückgebildeten Winterstadium entwickeln, ein langsamer Wachstumsprozess, stimuliert durch den saisonalen Anstieg der Tageslänge. Die Untersuchung von Fortpflanzungshormonen und Gonadenwachstum vor der Legeperiode unter kontrollierten Bedingungen zeigte, dass moderate Temperaturunterschiede oder Temperaturmuster das Timing dieser physiologischen Veränderungen nicht beeinflussten. Das Fehlen einer Temperaturregulation dieses Prozesses könnte in der Zukunft ein weiteres Vorverlagern der Legeperiode in einem wärmeren Klima potentiell unterbinden. Weil das Timing des Follikelwachstums im Eierstock scheinbar zum Teil genetisch bedingt ist, ist eine Anpassung durch früheres Gonadenwachstum voraussichtlich möglich. Allerdings zeigten Weibchen eine erstaunliche Plastizität im Legezeitpunkt des ersten Eies unabhängig vom Entwicklungsstand der Gonaden im späten Frühjahr. Dies impliziert dass zur Zeit physiologische Hemmnisse nicht für die natürlich vorhandene Variation im Legebeginn verantwortlich sind. Die Tatsache, dass eine Photostimulation wilder Kohlmeisenweibchen im Frühjahr, wodurch beschleunigtes Gonadenwachstums zu erwarten wäre, nicht zu einem früherem Legebeginn führte, bestätigt diese Sichtweise.

Die Größe der Eier variiert natürlicherweise zwischen Kohlmeisenweibchen, doch selbst innerhalb eines Geleges. Die Eigröße steigt im Frühjahr mit der Zeit geringfügig an, assoziiert mit dem Anstieg der Durchschnittstemperatur, doch auch mit dem veränderten Nahrungsspektrum. Im Gegensatz dazu wurden unter kontrollierten Bedingungen und unbegrenzter Nahrungszufuhr geringfügig größere Eier unter kälteren Bedingungen gelegt. Demzufolge ist es wahrscheinlicher, dass der saisonale Anstieg der Eigröße, was ein verbreitetes Muster bei kleinen Singvögeln ist, durch das Nahrungsspektrum und potentielle indirekte Temperatureinflüsse auf den Energiemetabolismus hervorgerufen wird.

Den Einfluss des Klimawandels auf die Populationsviabilität kleiner Singvögel vorherzusagen ist eine komplexe Aufgabe, besonders wenn man den hier vorgestellten Einfluss des Temperaturanstiegs auf saisonales Timing der Fortpflanzung berücksichtigt. Die Phänologie der Nahrungskette zwischen Eichen, Raupen und Kohlmeisen wird maßgeblich durch die Umgebungstemperatur bestimmt. Allerdings beeinflussen unterschiedliche Temperaturen verschiedene Komponenten dieser Nahrungskette zu unterschiedlichen Zeiten im Frühjahr. Selbst miteinander verbundene Life-history-Stadien von Vögeln sind zu einem größeren oder geringeren Ausmaß unter dem Einfluss von Temperatursignalen auf die saisonale Umwelt abgestimmt: Während der Legebeginn durch das Muster des Temperaturanstiegs bestimmt wurde, war dies für die zugrundeliegende Fortpflanzungsphysiologie oder den Beginn der Mauser im Spätsommer nicht der Fall. Ein Divergieren phänologischer Zusammenhänge ist infolgedessen wahrscheinlich. Die physiologische Basis saisonalen Timings zu untersuchen kann uns helfen Verlagerungen in der Dynamik von Nahrungsnetzen zu verstehen, die potentiell Artenabundanzen und Zusammensetzungen von Biozönosen mitbestimmen können.

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Affiliations of co-authors

Samuel P. Caro

Peter P. de Vries

Kees van Oers

Carolina Rueda

Luc te Marvelde

Marcel E. Visser

Department of Animal Ecology, Netherlands Institute of Ecology (NIOO-KNAW)
P.O. Box 50, 6700 AB Wageningen, The Netherlands

Gregory F. Ball

Department of Psychological and Brain Sciences,
Johns Hopkins University, 3400 North Charles Street, Baltimore, MD 21218, USA

Alistair Dawson

Centre for Ecology and Hydrology
Bush Estate, Penicuik, Midlothian EH26 0QB, United Kingdom

Phillip Gienapp

Ecological Genetics Research Unit, Department of Biosciences
University of Helsinki, Viikinkaari 11, FIN-00014 Helsinki, Finland

Barbara Helm

Max Planck Institute for Ornithology, 82346 Andechs, Germany

Peter J. Sharp

Roslin Institute and Royal (Dick) School of Veterinary Studies
University of Edinburgh, Edinburgh, Midlothian EH25 9RG, United Kingdom

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Curriculum vitae

Sonja Schaper was born on the 28th of February 1980 in Bad Oldesloe, Germany. After completing her secondary education she worked for one year as a research assistant on benthic fauna of the Wadden Sea in a governmental institute (Niedersächsisches Landesamt für Ökologie, Forschungsstelle Küste) in Wilhelmshaven, Germany. She started her studies of biology in 1999 at the University of Bremen, Germany, and later also at the University of Otago, Dunedin, New Zealand. During her time in New Zealand she participated in a reintroduction project on the rarest wading bird of the world, the Kaki (*Himantopus novaezelandiae*). She graduated from Bremen in December 2006 with specializations in ecology, zoology and marine biology. For her diploma thesis Sonja joined the workgroup Multitrophic Interactions at the Netherlands Institute of Ecology (NIOO-KNAW). Under supervision of Thomas Hoffmeister, University of Bremen and Jeff Harvey, NIOO she investigated plant-mediated above-belowground interactions in *Brassica nigra* in a field experiment. After her graduation she stayed shortly as a research assistant at the NIOO to investigate oviposition decisions in *Pieris* butterflies. Sonja then worked as a freelancer in nature conservation for "Stiftung NordWest Natur". Besides conceptual work on the water framework directive and Natura 2000 projects she collected avifaunistic data in the nature reserve Borgfelder Wümmewiesen. In January 2008 she started her PhD under supervision of Marcel Visser at the Animal Ecology group as part of the project "Adapting to a warmer world: phenology, physiology and fitness". This work on the effects of ambient temperature on seasonal timing of reproduction in great tits was completed in spring 2012 and is the subject of the present thesis.